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DATE: Monday, February 10, 2003

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DB=USPT,PGPB,JPAB,EPAB,DWPI; PLUR=YES; OP=ADJ			
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L3	kushner-p$.in.	66	L3
L2	L1 and (apl or ap-1 or fos or jun)	1018	L2
L1	nuclear$ near5 receptor$	2521	L1

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Database: All Databases (USPT + EPAB + JPAB + DWPI + TDBD)

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Search History

DB Name	Query	Hit Count	Set Name
ALL	l1 and l2	43	L4
ALL	progestin$ and ap-1	8	L3
ALL	glucocorticoi$ and ap-1	109	L2
ALL	#S137	59	L1

Search Results - Record(s) 1 through 8 of 8 returned.

1. Document ID: US 5990078 A

Entry 1 of 8 File: USPT Nov 23, 1999

US-PAT-NO: 5990078
DOCUMENT-IDENTIFIER: US 5990078 A

TITLE: Means of increasing estrogen receptor levels in neural tissue
DATE-ISSUED: November 23, 1999

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Toran-Allerand; C. Dominique	New York	NY	N/A	N/A

US-CL-CURRENT: 514/2; 514/12, 514/182, 530/399, 540/114, 540/117, 540/2

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Image

2. Document ID: US 5916910 A

Entry 2 of 8 File: USPT Jun 29, 1999

US-PAT-NO: 5916910
DOCUMENT-IDENTIFIER: US 5916910 A

TITLE: Conjugates of dithiocarbamates with pharmacologically active agents and uses therefore
DATE-ISSUED: June 29, 1999

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Lai; Ching-San	Encinitas	CA	N/A	N/A

US-CL-CURRENT: 514/423; 514/514, 548/564, 548/573, 558/235

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Image

3. Document ID: US 5869539 A

Entry 3 of 8 File: USPT Feb 9, 1999

US-PAT-NO: 5869539
DOCUMENT-IDENTIFIER: US 5869539 A

TITLE: Emulsions of perfluoro compounds as solvents for nitric oxide (NO)
DATE-ISSUED: February 9, 1999

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Garfield; R. E.	Friendswood	TX	N/A	N/A
Balaban; A. T.	Bucharest	N/A	N/A	ROX
Seitz; W. A.	Dickinson	TX	N/A	N/A

US-CL-CURRENT: 514/746; 424/673, 424/718, 514/743, 514/759, 514/832, 514/833

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Image

4. Document ID: US 5830848 A

Entry 4 of 8 File: USPT Nov 3, 1998

US-PAT-NO: 5830848
DOCUMENT-IDENTIFIER: US 5830848 A

TITLE: Method and agents for inducement of endogenous nitric oxide synthase for control and management of labor during pregnancy
DATE-ISSUED: November 3, 1998

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Harrison; Michael R.	San Francisco	CA	N/A	N/A
Heymann; Michael A.	San Francisco	CA	N/A	N/A
Riemer; Robert Kirk	Half Moon Bay	CA	N/A	N/A
Natuzzi; Eileen Stack	San Francisco	CA	N/A	N/A

US-CL-CURRENT: 514/2; 424/85.1, 424/85.2, 424/85.5, 530/399

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Image

5. Document ID: US 5723291 A

Entry 5 of 8 File: USPT Mar 3, 1998

US-PAT-NO: 5723291
DOCUMENT-IDENTIFIER: US 5723291 A

TITLE: Methods for screening compounds for estrogenic activity
DATE-ISSUED: March 3, 1998

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Kushner; Peter	San Francisco	CA	N/A	N/A
Webb; Paul	San Francisco	CA	N/A	N/A
Williard; Renee	San Francisco	CA	N/A	N/A
Hunt; C. Anthony	San Francisco	CA	N/A	N/A
Lopez; Gabriella	San Francisco	CA	N/A	N/A

US-CL-CURRENT: 435/6; 435/29, 435/69.1, 435/7.1, 435/7.2, 435/7.21, 435/7.23, 436/501, 536/24.1

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Image

6. Document ID: US 5686116 A

Entry 6 of 8 File: USPT Nov 11, 1997

US-PAT-NO: 5686116
DOCUMENT-IDENTIFIER: US 5686116 A

TITLE: Methods of enhancing repair, healing and augmentation of bone implants
DATE-ISSUED: November 11, 1997

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Bockman; Richard	New York	NY	N/A	N/A
Guidon; Peter	Cranford	NJ	N/A	N/A

US-CL-CURRENT: 424/650; 514/492, 514/8

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Image

7. Document ID: US 5639616 A

Entry 7 of 8 File: USPT Jun 17, 1997

US-PAT-NO: 5639616
DOCUMENT-IDENTIFIER: US 5639616 A

TITLE: Isolated nucleic acid encoding a ubiquitous nuclear receptor
DATE-ISSUED: June 17, 1997

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Liao; Shutsung	Chicago	IL	N/A	N/A
Song; Ching	Durham	NC	N/A	N/A

US-CL-CURRENT: 435/7.1; 435/252.3, 435/320.1, 435/69.1, 536/23.5, 536/24.3

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Image

8. Document ID: US 5310759 A

Entry 8 of 8 File: USPT May 10, 1994

US-PAT-NO: 5310759
DOCUMENT-IDENTIFIER: US 5310759 A

TITLE: Methods of protecting and preserving connective and support tissues
DATE-ISSUED: May 10, 1994

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Bockman; Richard S.	Sag Harbor	NY	11963	N/A

US-CL-CURRENT: 514/573

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Image

Term	Documents
PROGESTIN$	0
PROGESTIN	857
PROGESTINAL	5
PROGESTINATION	1
PROGESTINBASED	2
PROGESTINC	1
PROGESTINE	10
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PROGESTING	1
(PROGESTIN$ AND AP-1).ALL.	8

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Document Number 1

Entry 1 of 8 File: USPT Nov 23, 1999

DOCUMENT-IDENTIFIER: US 5990078 A
TITLE: Means of increasing estrogen receptor levels in neural tissue

DEPU:
113. MURAD F., KURET, J. A. (1990) Estrogens and Progestins. Goodman and Gilman's, the Pharmacolocrical Basis of Therapeutics, Gilman, A. G., et al., eds., Pergamon Press, pp. 1384-1396.

DEPU:
50. WEISZ, A. and ROSALES, R. (1990). Identification of an estrogen reponse element upstream of the human c-fos gene that binds the estrogen receptor and the AP-1 transcription factor. Nucleic Acids Res. 18:5097-5106.

Document Number 5

Entry 5 of 8 File: USPT Mar 3, 1998

DOCUMENT-IDENTIFIER: US 5723291 A
TITLE: Methods for screening compounds for estrogenic activity

DRPR:
FIGS. 2A-2C show antiestrogen stimulation of expression in an intact AP-1 Site, but not at classical EREs. FIG. 2A shows CAT assays of HeLa cells transfected with the indicated reporter genes and 3 .mu.g of human ER expression vector. Representations, at left, show the human collagenase promoter (shaded) and the consensus AP-1 site. CAT activities were from cells maintained in the absence of hormone or saturating concentrations of ICI 164,384 (ICI, 1 .mu.M), tamoxifen (5 .mu.M) or estradiol (100 nM). CAT activity is normalized to a transfection control with the actin promoter driving expression of .beta.HCG. Single representative experiments are shown, error bars represent standard deviation of triplicate hormone treatments. FIG. 2B shows CAT assays of HeLa cells transfected with reporter genes consisting of sequences overlapping the collagenase AP-1 site (-73 to -52) upstream of the herpes simplex virus TK promoter (from -109 to +45 relative to the start site of transcription) or the native TK promoter alone. FIG. 2C shows CAT assays of HeLa cells transfected with reporter genes containing classical EREs.

DRPR:
FIGS. 3A and 3B show that antiestrogen agonism at AP-1 sites requires ER. FIG. 3A shows dose dependence of estrogen and antiestrogen induction of coll73-LUC in HeLa cells relative to input ER expression vector, normalized to constant input DNA with blank expression vector SG5. The luciferase assays were normalized to actin-HCG and were expressed relative to values that were obtained with the collagenase promoter in the absence of expression vector and hormone. A single representative experiment with triplicate points is shown. Error bars represent standard deviations. FIG. 3B shows concentration dependence of estrogen and antiestrogen induction of coll73-LUC in HeLa cells. HeLa cells were transfected with 5 .mu.g of ER expression vector and the collagenase promoter active upon a luciferase reporter gene. The cells were exposed to a range of concentrations of ligand. Error bars represent standard deviation of triplicate points.

DRPR:
FIGS. 5A-C show that hormone response at the AP-1 site requires AP-1 proteins. FIG. 5A shows potentiation of hormone responses in HeLa cells by Jun and Fos. Relative luciferase activities, normalized to HCG production, and calculated relative to collagenase expression in the absence of ER and hormones are presented. The errors represent standard deviations of three separate experiments. FIG. 5B shows effects of ER with and without transfected Jun and Fos on hormone induction of the collagenase promoter in F9 cells. Averages of five or six individual transfections are shown. FIG. 5C shows response of the collagenase promoter in F9 cells to increasing amounts of Jun, Fos, or their combination, in the absence of ER.

DRPR:
FIGS. 6A-C show that the DNA binding domain of ER is required for tamoxifen induction at an AP-1 site, but not required for estrogen induction. Reporters regulated by an AP-1 site (left panels), or an ERE (right panels) were introduced into Hela (FIG. 6A), CHO (FIG. 6B), or MDA453 cells (FIG. 6C), with 5 .mu.g, 100 ng and 1 .mu.g respectively of each expression vector for the ER derivative whose structure is indicated. The DNA binding domain is indicated with the striped box, the ligand binding domain (AF2) and the amino terminal (AFI) activation functions are marked. Results are presented

for coll73-LUC in HeLa and MDA453 cells and coll73-CAT in CHO cells. CAT and luciferase activities are calculated relative to those obtained with coll73-LUC or coll73-CAT with SG5 blank expression vector in the absence of hormones.

DRPR:
FIGS. 7A and 7B show that fusing an exogenous transactivation function to the ER increases activation at AP-1 sites. A luciferase reporter regulated by an AP-1 site (left panels) and a CAT reporter regulated by an ERE (right panels) were introduced into Hela (FIG. 7A), CHO (FIG. 7B) with expression vector for the ER derivative whose structure is indicated. CAT or Luciferase activities, normalized to HCG production are shown. Activator plasmids, are shown schematically at the left of the figure. The VP16 transactivation domain is represented as an oval. The GALA DNA binding domain is marked.

DRPR:
FIGS. 8A and 8B show that fusing an exogenous transactivation function to an ER derivative without the ligand binding domain potentiates gene expression mediated by an ERE but not by an AP-1 site. A luciferase reporter regulated by an AP-1 site (left panels) and a CAT reporter regulated by an ERE (right panels) were introduced into HeLa cells with expression vector for ER derivatives. CAT or Luciferase activities, normalized to HCG production, are shown.

DEPR:
Antiestrogens which block the indirect pathway can be used to supplement tamoxifen or other antiestrogens in the treatment or prevention of breast cancer and other diseases mediated by estrogen. These compounds function to eliminate estrogenic agonistic activity of antiestrogens. Second they may have uses by themselves. In particular, it may be advantageous to block some estrogen mediated pathological effects at indirect estrogen response elements while leaving the direct pathway active. Compounds that block the indirect pathway are useful as components of combined oral contraceptives (COC) containing estrogens and progestins. A triple COC, containing estrogens, progestins, and a blocking compound would allow estrogen, either in the formulation or endogenous to act at the classical response elements, but would block action at the indirect response elements. Thus, a triple COC functions as current COCs to prevent pregnancy, but may also provide protective effects against breast cancer.

DEPR:
All reporter genes described below have been modified by digestion with Eco0109 and NdeI to remove an AP-1 site in the backbone of pUC. Thus, Coll73 and Coll60 are formerly .DELTA.Coll73 and .DELTA.Coll60 (Lopez et al. Mol. Cell. Biol. 13:3042-930 (1993)). Coll73-LUC was constructed by cloning a BamHI/PvuII fragment, that spanned the luciferase transcription unit, from pMG3 into coll73, which had been digested with BamHI and Sinai to remove the CAT transcription unit. EREcoll60 and EREcoll73 was prepared by ligation of a consensus ERE (AGGTCACAGTGACCT SEQ ID NO: 1), into the HindIII site upstream of coll60 and coll73, respectively. All other reporter genes have been previously described (Webb et al. Mol. Endocrinol. 6:157-16725 (1992); and Lopez et al., supra).

DEPR:
The human collagenase gene, like other matrix metalloproteases, responds to AP1. The promoter from this gene contains a consensus AP-1 site located between -60 and -73 base pairs from the start of transcription. Angel, et al., Mol. Cell. Biol., 7: 2256-2266 (1987). To test whether an AP1 site could confer estrogen response the collagenase promoter was fused to the bacterial CAT gene (.DELTA.coll73) and transfected into Chinese Hamster Ovary cells that over-express ER (ERC1) Kushner, et al. Mol. Endocrinol. 4:1465-1473 (1990).

DEPR:
To further examine the effects of antiestrogens on the AP-1 directed pathway, reporter genes derived from the human collagenase promoter were transfected into HeLa cells. Both estrogen and antiestrogens activated the collagenase promoter in the presence of transiently expressed human ER (coll517, FIG. 2A). In these cells tamoxifen was more potent an activator than estrogen. This pattern was retained with coll73, but was lost with coll60 or was inactivated by point mutations (coll517mAP1).

DEPR:
When the collagenase AP-1 site was placed upstream of the herpes virus tk

promoter both tamoxifen and ICI were able to activate transcription, although this response was not as robust as with the native collagenase promoter (FIG. 1B). These results indicate that antiestrogens are agonists at the collagenase promoter and a heterologous promoter linked to AP-1. Thus, the AP-1 site is required for this activity.

DEPR:
The activity of antiestrogens in the AP-1 pathway was also compared with their activity in the classical pathway. Direct substitution of an ERE for the collagenase AP-1 site restored estrogen response to the core collagenase promoter, but not antiestrogen response or the basal activity associated with the AP-1 site. A promoter with both an ERE and an AP-1 site (ERE-coll73 FIG. 2C) gave a large estrogen response, but retained some response to antiestrogens. Another control reporter, in which the tk promoter was regulated by a classical ERE (ERE-tk FIG. 2C) was also activated by estrogen, but not by antiestrogens. Thus, a classical ERE cannot substitute for the AP- 1 site, indicating that the AP-1 site has a unique function in activation by antiestrogens.

DEPR:
The data above show that antiestrogens are agonists at the AP-1 driven collagenase promoter, but not at classical EREs, in HeLa and other cells. To test whether this pattern was widespread, the effect of estrogen and antiestrogens on the expression of reporter genes driven by either the native collagenase promoter, or a similar promoter in which the AP-1 site was replaced by a classical ERE, was tested in a range of cell lines. In each case, the cells were transfected with different amounts of the human ER expression vector HEO to determine the optimal response.

DEPR:
Table I shows that both estrogen and antiestrogens activated the collagenase promoter in most cell types. This response occurred with cell lines representative of different tissue types including cervix, liver, myometrium, neuroblastoma, kidney and ovary. In most cases tamoxifen was as potent, or more potent, than estrogen. Only F9 cells, which have low levels of endogenous AP-1 activity, were not activated by any ligand.

DEPR:
In the same range of cell types both antiestrogens displayed little activity at classical EREs (data not shown). ICI consistently behaved as a pure antagonist of ER action at an ERE. In HeLa cells, and most other cases, tamoxifen inductions of ERE-coll60 activity remained at less than 3% of those obtained with estrogen. Significant (30% of estrogen) tamoxifen inductions at classical EREs were obtained in CEF cells and CV-1 cells and MDA453 cells. In these latter cells tamoxifen action at the AP-1 site was relatively weak (Table I). Thus, tamoxifen activity at an AP-1 site may be strong in cells at which its activity at an ERE is weak (HeLa), and weak at an AP-1 site in cells at which its activity at an ERE is strong (MDA453).

DEPR:
In conclusion, antiestrogen agonist effects occur at AP-1 sites in cells of diverse origin. These effects show little correlation with the activity of tamoxifen at classical EREs.

DEPR:
The data in Table I show that, in most cell types, tamoxifen was at least as potent as estrogen in inducing the collagenase promoter. In one cell line, MDA453 breast cancer cells, the AP-1 driven collagenase promoter was activated efficiently by estrogen but not by tamoxifen. Similarly, in Chinese hamster ovary (CHO) cells, estrogen inductions routinely exceeded antiestrogen inductions. This suggests that tamoxifen action at the collagenase promoter might have a cell specific component.

DEPR:
To test whether AP-1 proteins, as well as their cognate binding site, were required for the AP-1 pathway, we examined whether Jun and Fos overexpression affected the hormone response of the collagenase promoter. The Examples above establish that antiestrogens and estrogens activate the collagenase promoter in HeLa cells in the presence of ER (see, e.g., Table I). FIG. 5A shows that these inductions are markedly increased by the presence of transfected AP-1, especially in the presence of Jun or Jun/Fos. This suggests that Jun homodimers or Jun/Fos heterodimers occupying the AP-1 site contribute to the ability of ER to activate description in the AP-1 directed pathway.

DEPR:
To confirm that AP-1 proteins were absolutely required for the AP-1 directed ER pathway, we turned to F9 cells, which have only low levels of endogenous AP-1 activity. Transfection of an expression vector for estrogen receptor into these cells did not support hormone activation of the collagenase promoter (Table D, whereas it gave strong estrogen activation at an ERE (not shown). Co-transfection of ER with Jun/Fos restored induction by both estrogen and antiestrogens in F9 cells, albeit at lower levels than that seen in HeLa cells. In addition there was some activation by unliganded ER. Thus, the inability of F9 cells to allow a hormone response at the collagenase promoter can be overcome with AP-1 supplied by transfection. We conclude that hormone effects at the AP-1 site require AP-1 protein. However, the dramatic difference between the hormone response of HeLa and F9 cells when both are supplied with Jun and Fos indicates that other cell specific factors, in addition to AP-1 abundance, regulate the strength of the AP-1 directed ER pathway.

DEPR:
It is unlikely that ER dependent activation at AP-1 sites is due to changes in the mount of AP-1. In these studies we determined the mounts of AP-1 required for optimal collagenase promoter activity in F9 cells. FIG. 5C shows that Jun, Fos, and a combination of both, increased basal activity of the collagenase promoter (in the absence of ER) which reached a maximum with 300 ng of expression vector. These mounts were employed in the co-transfections with ER (FIG. 5B). Thus, ER activation at AP-1 sites appears to increase the transcriptional efficiency of Jun and Fos even when they are provided at optimal mounts.

DEPR:
To test whether ER effects upon AP-1 might reflect direct biochemical interaction between the ER and AP-1 proteins, we examined whether they specifically interact in solution. An estrogen receptor protein fused to glutathione S-transferase (ER-GST), and attached to agarose beads, pelleted in vitro translated Jun from solution, whereas a control GST protein pelleted only background amounts of Jun. Similar binding occurred with the ER amino terminal domain, but not with the LBD. Neither the intact ER nor its isolated domains bound Fos. These results indicate that Jun, but not Fos, binds ER in vitro, and that a major target of Jun is the ER amino terminus.

DEPR:
We next examined which domains of the ER mediate hormone action. We introduced truncated derivatives of the ER into three different cell types. We chose the HeLa, CHO and MDA453 lines as recipients because the ER driven AP-1 pathway showed different properties in each cell. In HeLa cells tamoxifen response predominated, in MDA453 cells estrogen response predominated, and CHO cells gave an intermediate phenotype (Table I). We examined the ability of each truncated ER to activate a reporter gene driven by the collagenase promoter with its AP-1 site (FIG. 6, left side) or a reporter gene driven by control promoter with an ERE (FIG. 6, right side). Previous work has established that each of these variant ERs is expressed at comparable levels from these vectors.

DEPR:
Deletion of the DNA binding domain (DBD) completely eliminated estrogen activation at an ERE in all three cell types (HE11, FIG. 6). Deletion of the DNA binding domain also eliminated tamoxifen activation at AP-1 sites, be it the substantial tamoxifen activation in HeLa and CHO cells, or the marginal mount in MDA cells. In contrast, removal of the DBD did not abolish estrogen activation at the AP-1 site in any of the cell lines. Indeed, estrogen activation at the AP-1 site in CHO cells was equally strong with or without the ER DBD. This is consistent with previous observations that estrogen response at AP-1 sites shows independence of DNA binding in CEF. Thus, the requirement for the ER DBD varies according to the ligand, it is required for tamoxifen induction but not estrogen induction. We suggest below (Discussion) that the differential requirements for the ER DBD may indicate the existence of more than one pathway of ER action at AP-1 sites.

DEPR:
The ER amino terminus also played an important role in tamoxifen and estrogen activation at the AP-1 site. Although deleting of the amino terminus (HE19) did not eliminate activity upon the ERE regulated reporter in all three cell types, this deletion abolished the strong

tamoxifen-activation at the AP-1 site in HeLa cells and the weaker tamoxifen activation in CHO and MDA453 cells. Deletion of the amino terminus also markedly reduced estrogen activation at the AP-1 site in all three cell types.

DEPR:
A deletion of the ligand binding domain (HE15), leaving the amino terminus and DBD intact, gave a constitutively active receptor that was able to weakly activate at an ERE in all three cell lines. This receptor, however, showed highly potent activity at the AP-1 site in HeLa cells, which correlated with the levels of activity obtained with the tamoxifen liganded native ER. In contrast, HE15 was inactive in MDA453 cells and weak in CHO cells. Thus, the requirement of the ER amino terminus for AP-1 activation also shows cell type specificity, in a manner that correlates with the cells ability to support a tamoxifen response at the collagenase promoter. This again suggests that activation through AP-1 may occur through more than one mechanism.

DEPR:
One possible mechanism for ER activation at AP-1 sites is that the receptor might directly bind to the AP-1 complex at the promoter and from there influence transcription. A prediction of this model is that ER should be able to target heterologous transcriptional activation functions to an AP-1 regulated promoter.

DEPR:
In order to test this proposition, we examined the effects of linking the strong VP16 transcriptional activation domain to the amino terminus of the ER (V-ER). To monitor activity we used a luciferase reporter gene regulated by an AP-1 site and CAT reporter gene driven by an otherwise identical promoter with an ERE. The V-ER chimeric receptor gave markedly enhanced activation at an ERE in HeLa cells (FIG. 7A). It was activated both by estrogen and antiestrogens reflecting the ability of VP16 to override the need for AF-2 (see, Kumar et al. Cell 51:941-951 (1987)), and consistent with previous reports for this "super-receptor". In contrast, the super-receptor had little effect at the AP-1 site in HeLa. Tamoxifen activation with the full length ER was hardly increased, although estrogen activation was modestly potentiated. We also tested a version of the super-receptor in which the ER DBD was deleted (VER.DELTA.DBD). This receptor, as expected, failed to activate at an ERE. It was, however, more potent than an equivalent ER (HE11) that lacked the VP16 activation function when tested at an AP-1 site.

DEPR:
To further explore this phenomenon we performed a series of similar experiments in CHO cells (FIG. 7B), in which estrogen response at the AP-1 site was completely independent of the ER DBD (FIG. 6B). Once again, the V-ER chimera superactivated gene expression that was driven by the ERE. In this case, however, V-ER also superactivated at the AP-1 site. Although the superreceptor that lacked the DBD (VER.DELTA.DBD) remained unable to activate transcription from an ERE, it was even more active than V-ER at the AP-1 site. A control fusion of the VP16 domain to the yeast GAL4 DNA binding domain did not increase collagenase promoter transcription. Thus, the superactivation by VP16 in CHO cells is dependent upon sequences in the ER protein. These observations indicate that super-receptors are super-activators at AP-1 sites in CHO cells in a DBD-independent manner. Similar results were also obtained with MDA453 cells (data not shown). The contrast between the properties of the super-ER in HeLa and CHO cells further suggests that there may be more than one pathway of activation at AP-1 sites.

DEPR:
The results described above suggest that addition of the VP16 activation function to native ER was unable to strongly potentiate tamoxifen action at an AP-1 site in HeLa cells. We also observed that an ER lacking the LBD (HE15) was a potent constitutive activator of the AP-1 pathway in HeLa, and that this correlated with the ability of these cells to support a large tamoxifen response at the collagenase promoter. To directly test whether transcriptional activation functions were involved in this pathway, we examined the effects of fusing the VP16 activation domain to this receptor (V-ER302C, FIG. 8). FIG. 8B shows that the presence of the VP16 domain greatly potentiated transcription from an ERE, but failed entirely to potentiate transcription activation by ER from the AP-1 site (FIG. 8A). Indeed, the presence of the VP16 domain slightly decreased the activity of

HE15 at the AP-1 site. Similar results were also obtained in CHO cells (data not shown). Thus the activation pathway of the LBD deleted receptor at AP-1 sites appears not to respond to exogenous transcriptional activation functions. We argue below that this suggests the existence of an ER pathway that activates transcription from AP-1 sites independent of ER associated transcriptional activation functions.

DEPU:
Antiestrogens Are Agonists of the AP-1 Pathway in Many Cell Types

DEPU:
Antiestrogens are Agonists of the AP-1 Pathway In Endometrial Cell Lines, But Not in Breast Cells

DEPU:
AP-1 Proteins Are Required for ER Action at the Collagenase Promoter

DEPU:
Tamoxifen Activation at AP-1 Requires the ER DBD, Whereas Estrogen Activation Is DBD Independent In Some Cell Types.

DEPU:
VP16 Potentiates the Action of an ER Without an LBD at an ERE, But Not at an AP-1 Site.

Document Number 7

Entry 7 of 43 File: USPT Dec 21, 1999

DOCUMENT-IDENTIFIER: US 6004748 A
TITLE: Method of inhibiting transcription utilizing nuclear receptors

ABPL:
This invention provides a method of inhibiting the transcription of a gene, which is activated by AP-1 or an AP-1 component, comprising binding AP-1 or the component with a nuclear receptor so as to prevent the binding of AP-1 to the gene. The nuclear receptor can be the retinoic acid receptor, glucocorticoid receptor, vitamin D3 receptor, thyroid receptor, or estrogen receptor. Also provided is a composition of matter comprising AP-1 or an AP-1 component bound to a nuclear receptor. These methods and compositions can be used to treat arthritis and cancer.

BSPR:
Since glucocorticoids and RA are known to repress members of the collagenase family they have promise as therapeutic agents in rheumatoid arthritis where proteinases, such as collagenase and stromelysin, play an important role in joint destruction. Collagenase is the only enzyme known to cleave collagen, a major structural component of bone and cartilage destroyed by the enzyme. Retinoids inhibit the production of collagenase by synovial cells while the tumor promoter 12-0-tetradecanoylphorbol-13-acetate (TPA) and the inflammatory mediators interleukin 1 (IL1) and tumor necrosis factor alpha (TNF.alpha.) stimulate collagenase secretion and transcription.

BSPR:
Thus, while the RA and glucocorticoid nuclear receptors are known to repress members of the collagenase family, the mechanism of this repression was not known. A logical presumption was that the repression relates to the receptors known ability to bind DNA. However, the present invention provides the surprising discovery that these nuclear receptors actually inhibit transcription through a protein/protein interaction with AP-1, a protein complex composed of Jun homodimers and Jun/Fos heterodimers. Hence, a major discovery that regulatory function of nuclear receptors are mediated by a mechanism that does not involve direct binding to DNA is provided. This discovery provides a mechanism through which arthritis and cancer can be treated.

BSPR:
This invention provides a method of inhibiting the transcription of a gene, which is activated by AP-1 or an AP-1 component, comprising binding AP-1 or the component with a nuclear receptor so as to prevent the binding of AP-1 to the gene. The nuclear receptor can be the retinoic acid receptor, glucocorticoid receptor, vitamin D3 receptor, thyroid receptor, or the estrogen receptor.

BSPR:
Also provided is a composition of matter comprising AP-1 or an AP-1 component bound to a nuclear receptor. These methods and compositions can be used to identify and screen for new ligands of nuclear receptors useful for treatment of arthritis and cancer.

DRPR:
FIG. 4 shows inhibition of cJun binding by RAR.beta. and RAR.tau. on synthetic AP-1 site.

DEPR:
The invention provides a method of inhibiting the transcription of a gene,

which is activated by AP-1 or an AP-1 component, comprising binding of AP-1 or a component of it with a nuclear receptor so as to prevent the binding of AP-1 to the gene.

DEPR:
It is known that Jun and Fos protooncoproteins make up AP-1. Thus, in one embodiment, the AP-1 component is a Jun protein or a Fos protein or portions thereof which either individually or in combination with other components activate transcription through AP-1 responsive nucleotide sequences. Thus, by "AP-1" is meant any compound having the structure of "AP-1" necessary for the binding of AP-1 to its responsive element.

DEPR:
In addition, "nuclear receptor" means a receptor, such as retinoic acid receptor, glucocorticoid receptor, vitamin D3 receptor, thyroid receptor and estrogen receptor, or portions of these receptors, which retain the function of binding AP-1 or transcriptionally activating fragments of AP-1.

DEPR:
The gene in which transcription is inhibited can be any gene which is transcriptionally activated by AP-1 or an AP-1 component. In one embodiment, the gene encodes collagenase.

DEPR:
Since AP-1 is a transcriptional activator of the gene encoding collagenase, and collagenase is one of the enzymes known to break down collagen, a component of bone, the control of AP-1 mediated transcription can be utilized to treat arthritis. Likewise, since AP-1 is comprised of the protooncogene encoded products Jun and Fos, the control of AP-1 mediated transcription can be utilized to treat those cancers caused by AP-1, a Jun or Fos component or another oncogene that regualtes AP-1 activity. Examples of control of AP-1 mediated cancer include the overexpression of AP-1, the expression of mutated forms of AP-1 and the increased AP-1 activity caused by expression of oncogenes such as H-ras.

DEPR:
Typically, the nuclear receptor binds to its ligand, e.g. retinoic acid receptor to retinoic acid, prior to binding to AP-1 or an AP-1 component. Thus, the invention provides methods in which ligand/receptor binding is required. However, circumstances can exist where the receptor directly binds to AP-1 or an AP-1 component.

DEPR:
Applicant's discovery also provides a novel composition of matter comprising AP-1 or an AP-1 component bound to a nuclear receptor. Examples of the nuclear receptor include retinoic acid, glucocorticoid, vitamin D3, thyroid and estrogen. Retinoic acid receptor includes RAR.epsilon., RAR.alpha. and RAR.tau. and thyroid receptor includes erbA-T, TR.alpha.-2 and TR.alpha.-2 variant.

DEPR:
The invention also provides a method of promoting the transcription of a gene which is activated by AP-1 or an AP-1 component comprising preventing the binding of AP-1, or an AP-1 component, with a nuclear receptor thereby allowing AP-1 to bind the gene. Such a method, given the teaching of the subject application, could be carried out by a person skilled in the art.

DEPR:
The invention still further provides a method of screening a sample for ligands which bind to a nuclear receptor to form a complex which binds or interferes with AP-1 or an AP-1 component. The method comprises contacting the ligand receptor complex with AP-1 and determining the binding of the complex to AP-1, the presence of binding or interference with AP-1 activity indicating the presence of a ligand. The binding of the complex to AP-1 can result in an increased anti-cancer or anti-arthritis effect compared to a ligand known to bind a nuclear receptor. Thus, one can screen for ligands with increased specificity or affinity of the receptor/ligand complex for AP-1 or AP-1 components. These ligands can be made by standard organic synthesis and screened using the methods of the invention.

DEPR:
This invention provides the added discovery that there is a family of nuclear receptors, in addition to their function as DNA binding transcriptional activators, which regulate transcription through protein

interactions, especially with AP-1. Thus, the invention is much more than individual receptors binding AP-1. The invention involves the discovery that the family of nuclear receptors can act as transcriptional regulators through protein/protein interactions.

DEPR:
The region of the collagenase promoter that confers repression by RA is located between residues -73 and -63, which contain the AP-1 site that is responsible for induction by TPA, TNF.alpha. and IL1 (Angel et al., Mol. Cell. Biol. 7:2256-2266 (1987a); Brenner et al., Nature 337:661-663 (1989); Lafyatis et al., Mol. Endo. 4:973 (1990)). In addition, this site confers repression by GR as shown in Example VI.

DEPR:
When the -73Col-CAT construct was transfected into Hela cells, grown in the presence and absence of RA, no substantial RA-dependent decrease in its basal or TPA induced CAT activity was observed. However, when an RAR.beta. expression vector was co-transfected with the -73Col-CAT construct, a dramatic repression of CAT activity was observed in the presence of RA, but not in the absence of RA. This repression was observed in either TPA-treated (FIG. 1) or untreated cells. Co-transfection of RAR.beta. with the -63Col-CAT showed no effect in the presence or absence of RA. The repression of -73Col-CAT by RAR.beta. is specific because the activity of another TPA-inducible promoter, that of cFos was not affected. Exchanging the RAR.beta. expression vector for the RAR.tau. vector yielded similar results. The repression of the TPA induced activity of -73 Col-CAT by RAR.beta. is concentration dependent: half maximal inhibition occurred at 10.sup.-9 M RA, while maximal repression required 10.sup.-6 M RA (FIG. 1). A similar concentration dependence was found for activation of an RAR.epsilon. reporter gene by RAR.beta.. Thus, the repression of collagenase transcription by RA is mediated by the AP-1 site and dependent on the level of RA activated RAR. This implies that the RARs either function by direct interaction with the AP-1 site and thereby interfere with AP-1 binding through a competitive mechanism, or alternatively by a mechanism that does not require direct binding of the RAR to the AP-1 site, but involves RAR-mediated interference with AP-1 activity. Despite a previous report that RA may repress collagenase expression by inhibition of cFos induction (Lafyatis et al., 1990 supra), we have not been able to find any effect of RA and RARs on the cFos promoter, in either HeLa (FIG. 1) or F9 cells.

DEPR:
To further demonstrate the involvement of AP-1 in repression of collagenase transcription by RA, we transfected the -73 Col-CAT reporter into F9 cells which have very low endogenous AP-1 activity. Co-transfection of -73 Col-CAT with a cJun expression vector activates its transcription, while a cFos expression vector does not lead to significant activation. As previously shown (Chiu et al., Cell 54:541-552 (1988)) co-transfection with a combination of cJun and cFos expression vector leads to further activation of -73 Col-CAT expression. In either case, expression of -73 Col-CAT was inhibited by RA, and further inhibition was observed in the presence of co-transfected RAR.beta. expression vector. Thus, elevating the level of RAR expression in F9 cells increases the repression of AP-1 activity by RA. Inhibition occurs regardless whether AP-1 activity is due to cJun homodimers or cJun/cFos heterodimers.

DEPR:
Next, we investigated whether the functional antagonism between RAR and AP-1 activity is limited to the AP-1 target site or whether it can be extended to targets which are positively regulated by RARs.

DEPR:
Co-transfection of the reporter T3RE.sub.2 -CAT that contains two RAR responsive elements in front of the HSV-TK promoter (Glass et al., Cell 54:313-323 (1988)), with increasing concentrations of either a cJun or a cFos expression vector, resulted in strong dose-dependent inhibition of its activation by RA (FIG. 2). Similar inhibition has also been obtained when a CAT reporter gene containing the RARE derived from the RAR.beta. promoter was used. Co-transfection of T3RE.sub.2 -CAT with cFos expression vector resulted in a more effective inhibition of its induction than a co-transfection with a cJun expression vector. No further inhibition was obtained by co-transfection with a combination of the cJun and cFos expression vectors. Hence, elevated levels of both AP-1 constituents can repress the activation of an RARE by RAR.

DEPR:
Recently, it was reported that AP-1 may repress osteocalcin induction by RA and vitamin D by binding to a site embedded within an RA and vitamin D response element (Schule et al., 1990a). We therefore investigated the possibility that RAR can bind specifically to the AP-1 site or neighboring sequences of the collagenase promoter.

DEPR:
In vitro synthesized glucocorticoid receptor (GR) and retinoic acid receptor (RAR) were incubated with .sup.32 P-labelled DNA fragment derived from -73 COL-CAT by Hind III and Bam HI digestion. Protein-DNA complex was analyzed by gel retardation assay. Control represents the binding of unprogrammed reticulocyte lysate.

DEPR:
Gel retardation experiments were carried out with a 60 bp DNA fragment containing the collagenase AP-1 site. While specific binding of cJun to this site (FIG. 3) or a synthetic AP-1 site was readily detected, RAR.beta. did not bind to either site (FIG. 3). As reported in Example VI, GR also did not bind to either probe (FIG. 3). Thus, the binding of RAR to site overlapping the AP-1 site cannot account for repression of collagenase induction, neither does the AP-1 site constitute a minimal RAR recognition element as previously suggested (Schule et al., supra).

DEPR:
As an explanation for its ability to repress AP-1 activity, we examined the effect of RAR on AP-1 binding activity.

DEPR:
When cell-free translated RAR.beta. or RAR.tau. proteins were mixed with bacterially produced or in vitro translated cJun, a significant inhibition of cJun binding to the AP-1 site was seen (FIG. 4). The Inhibition of cJun binding to the AP-1 site was dependent on the amount of RARs used, and was also observed in response to pre-incubation with in vitro synthesized GR (FIG. 4). As shown for the GRs in Example VI, this in vitro inhibition of cJun binding was ligand independent. The inhibition of Jun binding activity is a specific effect of each receptor because pre-incubation of the cell-free translated RAR.beta. with anti-RAR antibodies prevented this inhibition, while pre-incubation with anti-GR or preimmune serum had no effect. Likewise, the inhibitory activity of cell-free translated GR is inhibited by anti-GR antibodies, but not by anti-RAR or preimmune serum. Because of its increased stability, the cJun/cFos heterodimer exhibits stronger AP-1 binding activity than the cJun homodimer. We therefore investigated whether the binding of the heterodimers could also be inhibited by the RAR. DNA binding was strongly enhanced by the addition of in vitro synthesized cFos, while cFos by itself did not bind the AP-1 site. Addition of RAR resulted in a comparable degree of repression for both cJun and cJun/cFos binding to the AP-1 site.

DEPR:
The inhibition of cJun or cJun/cFos DNA binding by RAR results from an interaction between the two classes of proteins in which the DNA binding activities of both proteins are impaired. Additionally, AP-1 could interfere with RAR function by competing for RARE binding. We therefore also investigated whether cJun could bind to the RARE and/or inhibit RAR DNA binding.

DEPR:
In addition, applicants have discovered that the thyroid hormone receptor, in the presence of Thyroid hormone, binds AP-1 through a protein/protein interaction and inhibits the transcription of AP-1 activated genes. Specifically, TR.alpha. represses TPA induced collagenase promoter activity in a T3 dependent fashion. The -73 Col CAT reporter gene was cotransfected into CV-1 cells with an expression vector for RAR.beta./.epsilon.. Cells were grown in the presence of the indicated amounts of T3 and/or TPA. Repression of TPA collagenase promoter activity is dependant on the concentration of T3. These results are shown in FIG. 6.

DEPR:
Applicants have also shown that the glucocorticoid receptor (GCR) is a potent inhibitor of AP-1 activity (Jun/Fos) and both c-Jun and c-Fos are potent repressors of GCR activity. In vitro experiments using purified GCR and c-Jun proteins showed that mutual repression is due to direct interaction between the two. Direct interaction between GCR and either c-Jun

or c-Fos is demonstrated by cross-linking and coimmunoprecipitation. These findings also revealed a cross talk between two major signal transduction systems used to control gene transcription in response to extracellular stimuli, and a novel protein/protein interaction between the GCR and AP-1. The data demonstrating these findings is set forth in Yang-Yen et al., Cell 62:1205-1215, (1990).

DEPC:
The AP-1 Site of the Collagenase Promoter is Repressed by RARs

DEPC:
RAR Does Not Bind the AP-1 Site, But Inhibits Jun DNA Binding

DEPC:
Glucocorticoid Receptor Inhibits AP-1 Activity

CLPR:
1. A method of inhibiting the transcription of a gene, which is activated by AP-1 or an AP-1 component, comprising binding AP-1 or the component with a nuclear receptor so as to prevent the binding of AP-1 to the gene, wherein transcription is inhibited.

CLPR:
2. The method of claim 1, wherein the AP-1 component is a Jun protein.

CLPR:
3. The method of claim 1, wherein the nuclear receptor is a glucocorticoid receptor.

CLPR:
5. The method of claim 1, wherein the nuclear receptor is an estrogen receptor.

CLPR:
12. A method of inhibiting the transcription of a gene which is activated by a nuclear receptor which binds AP-1 or an AP-1 component comprising binding the receptor with AP-1 or an AP-1 component so as to prevent the binding of the nuclear receptor to the gene, wherein transcription is inhibited.

CLPR:
13. A composition of matter comprising AP-1 or an AP-1 component bound to a nuclear receptor.

CLPR:
14. The composition of claim 13, wherein the AP-1 component is selected from the group consisting of a Jun and a Fos protein.

CLPR:
15. The composition of claim 13, wherein the nuclear receptor is a glucocorticoid receptor.

CLPR:
17. The composition of claim 13, wherein the nuclear receptor is an estrogen receptor.

CLPR:
22. A method of promoting the transcription of a gene which is activated by AP-1 or an AP-1 component, comprising preventing the binding of AP-1 or an AP-1 component with a nuclear receptor thereby allowing AP-1 to bind the gene, wherein transcription is promoted.

ORPL:
Mordacq and Linzer, "Co-localization of Elements Required for Phorbol Ester Stimulation and Glucocorticoid Repression of Proliferin Gene Expression," Genes Dev. 3:760-769 (1989).

ORPL:
Offringa et al., "Similar Effects of Adenovirus E1A and Glucocorticoid Hormones on the Expression of the Metalloprotease Stromelysin," Nucl. Acids Res. 16:10973-10983 (1988).

ORPL:
Schule et al., "Functional Antagonism between Oncoprotein c-Jun and the Glucocorticoid Receptor," Cell 62:1217-1226 (1990).

ORPL:
Yang-Yen et al., "Transcriptional Interference between c-Jun and the Glucocorticoid Receptor: Mutual Inhibition of DNA Binding due to Direct Protein-Protein Interaction," Cell 62:1205-1215 (1990).

ORPL:
Yang-Yen et al., Transcriptional interference between c-Jun and glucocorticoid receptor: mutual inhibition of DNA-binding due to direct protein-protein interaction, Cell 62:6:1205-1215 (1990).

ORPL:
Jonat, C. et al., "Antitumor Promotion and Antiinflammation:Down-Modulation of AP-1 (Fos/Jun) Activity byG lucocorticoid Hormone," Cell 62:1189-1204 (1990).

ORPL:
Drouin et al., "Glucocorticoid receptor binding to a specific DNA sequence is required for hormone-dependent inhibition of pro-optomelanocortin gene transcription", Mol. Cell Biol., 9:5305-5314 (1989).

ORPL:
Offringa et al., "Similar effects of adenovirus EIA and glucocorticoid hormones on the expression of the metalloprotease stromelysin," Nucl. Acids Res., 16:10974-10983 (1988).

ORPL:
Mordacq, J.C. and Linzer, D.I.II., "Co-localization of elements required for phorbol ester stimulation and glucocorticoid repression of proliferin gene expression," Genes Dev., 3:760-769 (1989).

ORPL:
Yang-yen et al., Transcriptional interference between c-Jun and glucocorticoid receptor: mutual inhibition of DNA-binding due to direct protein-protein interaction, Cell, 62:6:1205-1215 (1990).

ORPL:
Schule et al., "Functional antagonism between oncoprotein c-Jun and the glucocorticoid receptor," Cell, 62:1217-1226 (1990).

ORPL:
Jonat et al., "Antitumor Promotion and Antiinflammation:Down-Modulation of AP-1 (Fos/Jun) Activity by Glucocorticoid Hormone," Cell, 62:1189-1204 (1990).

ORPL:
Drouin et al., "Glucocorticoid Receptor Binding to a Specific DNA Sequence is Required for Hormone-Dependent Repression of Pro-Opiomelanocortin Gene Transcription," Mol. Cell Biol. 9:5305-5314 (1989).

ORPL:
Jonat et al., "Antitumor Promotion and Antiinflammation: Down-Modulation of AP-1 (Fos/Jun) Activity by Glucocorticoid Hormone," Cell 62:1189-1204 (1990).

Search Results -

Term	Documents
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AP-1 AND 1	59

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Refine Search: `l1 and ap-1`

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ALL	l1 and l2	4	L3
ALL	kushner-p$.in.	50	L2
ALL	estrogen$ near5 receptor$	1528	L1

Search Results - Record(s) 1 through 10 of 59 returned.

1. Document ID: US 6046047 A

Entry 1 of 59 File: USPT Apr 4, 2000

US-PAT-NO: 6046047
DOCUMENT-IDENTIFIER: US 6046047 A

TITLE: Regulated transcription of targeted genes and other biological events
DATE-ISSUED: April 4, 2000

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Crabtree; Gerald R.	Woodside	CA	N/A	N/A
Schreiber; Stuart L.	Cambridge	MA	N/A	N/A
Spencer; David M.	Los Altos	CA	N/A	N/A
Wandless; Thomas J.	Cambridge	MA	N/A	N/A
Belshaw; Peter	Cambridge	MA	N/A	N/A
Ho; Steffan N.	San Diego	CA	N/A	N/A

US-CL-CURRENT: 435/320.1; 536/23.4, 536/23.5, 536/23.53

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Image

2. Document ID: US 6043082 A

Entry 2 of 59 File: USPT Mar 28, 2000

US-PAT-NO: 6043082
DOCUMENT-IDENTIFIER: US 6043082 A

TITLE: Regulated transcription of targeted genes and other biological events
DATE-ISSUED: March 28, 2000

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Crabtree; Gerald R.	Woodside	CA	N/A	N/A
Schreiber; Stuart L.	Cambridge	MA	N/A	N/A
Spencer; David M.	Los Altos	CA	N/A	N/A
Wandless; Thomas J.	Cambridge	MA	N/A	N/A
Ho; Steffan N.	San Diego	CA	N/A	N/A
Belshaw; Peter	Cambridge	MA	N/A	N/A

US-CL-CURRENT: 435/320.1; 536/23.4

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Image

3. Document ID: US 6037329 A

Entry 3 of 59 File: USPT Mar 14, 2000

US-PAT-NO: 6037329
DOCUMENT-IDENTIFIER: US 6037329 A

TITLE: Compositions containing nucleic acids and ligands for therapeutic treatment
DATE-ISSUED: March 14, 2000

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Baird; J. Andrew	San Diego	CA	N/A	N/A
Chandler; Lois Ann	Encinitas	CA	N/A	N/A
Sosnowski; Barbara A.	Coronado	CA	N/A	N/A

US-CL-CURRENT: 514/44; 424/93.21, 435/320.1, 435/325, 435/455, 435/458, 435/69.1, 530/350, 536/23.1

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Image

4. Document ID: US 6025388 A

Entry 4 of 59 File: USPT Feb 15, 2000

US-PAT-NO: 6025388
DOCUMENT-IDENTIFIER: US 6025388 A

TITLE: Method for inhibiting gene expression promoted by AP1 protein with RAR.beta. selective retinoids and method for treatment of diseases and conditions with such retinoids
DATE-ISSUED: February 15, 2000

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Nagpal; Sunil	Irvine	CA	N/A	N/A
Song; Tae K.	Long Beach	CA	N/A	N/A
Vuligonda; Vidyasagar	Irvine	CA	N/A	N/A
Athanikar; Jyoti	Irvine	CA	N/A	N/A
Chandraratna; Roshantha A.	Mission Viejo	CA	N/A	N/A

US-CL-CURRENT: 514/460; 514/336

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Image

5. Document ID: US 6025196 A

Entry 5 of 59 File: USPT Feb 15, 2000

US-PAT-NO: 6025196
DOCUMENT-IDENTIFIER: US 6025196 A

TITLE: Chimeric proteins comprising liver enriched transcription factors and nucleic acids encoding the same
DATE-ISSUED: February 15, 2000

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Sladek; Frances M.	Riverside	CA	N/A	N/A
Zhong; Weimin	New York	NY	N/A	N/A
Darnell, Jr.; James E.	Larchmont	NY	N/A	N/A

US-CL-CURRENT: 435/320.1; 435/69.7, 530/350, 536/23.4

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Image

6. Document ID: US 6011018 A

Entry 6 of 59 File: USPT Jan 4, 2000

US-PAT-NO: 6011018
DOCUMENT-IDENTIFIER: US 6011018 A

TITLE: Regulated transcription of targeted genes and other biological events
DATE-ISSUED: January 4, 2000

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Crabtree; Gerald R.	Woodside	CA	N/A	N/A
Schreiber; Stuart L.	Cambridge	MA	N/A	N/A
Spencer; David M.	Los Altos	CA	N/A	N/A
Wandless; Thomas J.	Cambridge	MA	N/A	N/A
Belshaw; Peter	Cambridge	MA	N/A	N/A

US-CL-CURRENT: 514/31; 424/93.21, 514/44, 514/9

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Image

7. Document ID: US 6008204 A

Entry 7 of 59 File: USPT Dec 28, 1999

US-PAT-NO: 6008204
DOCUMENT-IDENTIFIER: US 6008204 A

TITLE: Synthesis and use of retinoid compounds having negative hormone and/or antagonist activities
DATE-ISSUED: December 28, 1999

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Klein; Elliott S.	Marina del Rey	CA	N/A	N/A
Johnson; Alan T.	Rancho Santa Margarita	CA	N/A	N/A
Standeven; Andrew M.	Corona del Mar	CA	N/A	N/A
Beard; Richard L.	Newport Beach	CA	N/A	N/A
Gillett; Samuel J.	Albany	CA	N/A	N/A
Duong; Tien T.	Irvine	CA	N/A	N/A
Nagpal; Sunil	Lake Forest	CA	N/A	N/A
Vuligonda; Vidyasagar	Irvine	CA	N/A	N/A
Teng; Min	Aliso Viejo	CA	N/A	N/A
Chandraratna; Roshantha A.	Mission Viejo	CA	N/A	N/A

US-CL-CURRENT: 514/63; 514/544, 514/568, 514/569, 514/682, 556/437, 560/51, 562/462, 568/468

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Image

8. Document ID: US 6007991 A

Entry 8 of 59 File: USPT Dec 28, 1999

US-PAT-NO: 6007991
DOCUMENT-IDENTIFIER: US 6007991 A

TITLE: Antisense oligonucleotides for mitogen-activated protein kinases as therapy for cancer
DATE-ISSUED: December 28, 1999

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Sivaraman; Vimala S.	Setauket	NY	N/A	N/A
Wang; Hsien-yu	Setauket	NY	N/A	N/A
Malbon; Craig C.	Setauket	NY	N/A	N/A

US-CL-CURRENT: 435/6; 435/325, 514/44, 536/24.5

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Image

9. Document ID: US 6004748 A

Entry 9 of 59 File: USPT Dec 21, 1999

US-PAT-NO: 6004748
DOCUMENT-IDENTIFIER: US 6004748 A

TITLE: Method of inhibiting transcription utilizing nuclear receptors
DATE-ISSUED: December 21, 1999

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Pfahl; Magnus	Solana Beach	CA	N/A	N/A
Karin; Michael	San Diego	CA	N/A	N/A

US-CL-CURRENT: 435/6; 530/358, 530/359, 536/23.1, 536/24.1

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Image

10. Document ID: US 6004746 A

Entry 10 of 59 File: USPT Dec 21, 1999

US-PAT-NO: 6004746
DOCUMENT-IDENTIFIER: US 6004746 A

TITLE: Interaction trap systems for detecting protein interactions
DATE-ISSUED: December 21, 1999

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Brent; Roger	Cambridge	MA	N/A	N/A
McCoy; John M.	Reading	MA	N/A	N/A
Jessen; Timm H.	Bad Soden	N/A	N/A	DEX

US-CL-CURRENT: 435/6; 435/254.21, 435/325, 435/4

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Image

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51. Document ID: US 5580736 A

Entry 51 of 59 File: USPT Dec 3, 1996

US-PAT-NO: 5580736
DOCUMENT-IDENTIFIER: US 5580736 A

TITLE: Interaction trap system for isolating novel proteins
DATE-ISSUED: December 3, 1996

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Brent; Roger	Cambridge	MA	N/A	N/A
Gyuris; Jeno	Arlington	MA	N/A	N/A
Golemis; Erica	Somerville	MA	N/A	N/A

US-CL-CURRENT: 435/6; 435/254.2, 435/320.1, 435/69.1, 530/358, 536/23.5

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Image

52. Document ID: US 5580721 A

Entry 52 of 59 File: USPT Dec 3, 1996

US-PAT-NO: 5580721
DOCUMENT-IDENTIFIER: US 5580721 A

TITLE: Assays for inhibitors of myc oncoprotein
DATE-ISSUED: December 3, 1996

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Brent; Roger	Cambridge	MA	N/A	N/A
Golemis; Erica	Somerville	MA	N/A	N/A
Lech; Karen F.	Boston	MA	N/A	N/A
Anderson; Catherine	Cambridge	MA	N/A	N/A

US-CL-CURRENT: 435/6; 435/320.1, 530/358, 536/23.4

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Image

53. Document ID: US 5565334 A

Entry 53 of 59 File: USPT Oct 15, 1996

US-PAT-NO: 5565334
DOCUMENT-IDENTIFIER: US 5[redacted]334 A

TITLE: Enhancer sequence for modulating expression in epithelial cells
DATE-ISSUED: October 15, 1996

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Kufe; Donald	Wellesley	MA	N/A	N/A
Abe; Miyako	Boston	MA	N/A	N/A

US-CL-CURRENT: 435/69.1; 435/320.1, 435/371, 536/23.1, 536/23.2, 536/24.1, 536/24.5

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Image

54. Document ID: US 5556956 A

Entry 54 of 59 File: USPT Sep 17, 1996

US-PAT-NO: 5556956
DOCUMENT-IDENTIFIER: US 5556956 A

TITLE: Methods and compositions relating to the androgen receptor gene and uses thereof
DATE-ISSUED: September 17, 1996

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Roy; Arun K.	San Antonio	TX	N/A	N/A
Chatterjee; Bandana	San Antonio	TX	N/A	N/A

US-CL-CURRENT: 536/24.1; 536/23.1, 536/24.3, 536/24.31

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Image

55. Document ID: US 5514571 A

Entry 55 of 59 File: USPT May 7, 1996

US-PAT-NO: 5514571
DOCUMENT-IDENTIFIER: US 5514571 A

TITLE: Cyclin D1 negative regulatory activity
DATE-ISSUED: May 7, 1996

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Riabowol; Karl T.	Calgary	N/A	N/A	CAX

US-CL-CURRENT: 435/461; 435/6

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Image

56. Document ID: US 5512483 A

Entry 56 of 59 File: USPT Apr 30, 1996

US-PAT-NO: 5512483
DOCUMENT-IDENTIFIER: US 5[redacted]2483 A

TITLE: Expression vectors responsive to steroid hormones
DATE-ISSUED: April 30, 1996

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
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US-CL-CURRENT: 435/320.1; 536/24.1

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Image

57. Document ID: US 5445941 A

Entry 57 of 59 File: USPT Aug 29, 1995

US-PAT-NO: 5445941
DOCUMENT-IDENTIFIER: US 5445941 A

TITLE: Method for screening anti-osteoporosis agents
DATE-ISSUED: August 29, 1995

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US-CL-CURRENT: 435/6; 536/24.1

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Image

58. Document ID: WO 9207072 A1

Entry 58 of 59 File: EPAB Apr 30, 1992

PUB-NO: WO009207072A1
DOCUMENT-IDENTIFIER: WO 9207072 A1
TITLE: METHOD OF INHIBITING TRANSCRIPTION UTILIZING NUCLEAR RECEPTORS

PUBN-DATE: April 30, 1992

INVENTOR-INFORMATION:

NAME	COUNTRY
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INT-CL (IPC): C07K 13/00; C12N 15/00

EUR-CL (EPC): C12N015/67; G01N033/53, C07K014/705 , C07K014/72 , C07K014/82

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Image

59. Document ID: US 6004748 A, WO 9207072 A1, AU 9189469 A, EP 552302 A1, JP 06502532 W, EP 552302 A4, US 5643720 A

Entry 59 of 59 File: DWPI Dec 21, 1999

DERWENT-ACC-NO: 1992-167[redacted]
DERWENT-WEEK: 200006

TITLE: Method of inhibiting transcription of AP-1 activated genes - useful for the treatment of arthritis and cancer

INVENTOR: KARIN, M; PFAHL, M

PRIORITY-DATA:

1990US-0595582	October 10, 1990
1993US-0032726	March 16, 1993
1994US-0182735	January 14, 1994
1996US-0757349	November 27, 1996

PATENT-FAMILY:

PUB-NO	PUB-DATE	LANGUAGE	PAGES	MAIN-IPC
US 6004748 A	December 21, 1999	N/A	000	C12Q001/68
WO 9207072 A1	April 30, 1992	E	024	C12N015/00
AU 9189469 A	May 20, 1992	N/A	000	C12N015/00
EP 552302 A1	July 28, 1993	E	024	C12N015/00
JP 06502532 W	March 24, 1994	N/A	008	C12N015/63
EP 552302 A4	November 10, 1993	N/A	000	C12N015/00
US 5643720 A	July 1, 1997	N/A	011	C12Q001/68

INT-CL (IPC): A61K 37/02; A61K 45/00; C07H 21/04; C07K 13/00; C07K 14/705; C12N 15/00 ; C12N 15/63; C12Q 1/68; G01N 33/50

Term	Documents
AP-1	539
AP-1S	1
AP-1 AND 1	59

Document Number 47

Entry 47 of 59 File: USPT Jun 17, 1997

US-PAT-NO: 5639592
DOCUMENT-IDENTIFIER: US 5639592 A

TITLE: Functional antagonism between proto-oncoprotein c-Jun and hormone receptors

DATE-ISSUED: June 17, 1997

INVENTOR-INFORMATION:

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Schule; Ronald	Schopfheim	N/A	N/A	DEX

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APPL-NO: 8/ 030330
DATE FILED: May 3, 1994

PARENT-CASE:
This application is a 371 application of PCT/US91/06848, filed Sep. 20, 1991 and a continuation-in-part of application Ser. No. 07/586,187, filed Sep. 21, 1990, now abandoned.

PCT-DATA:
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PCT-APPL-NO: PCT/US91/06848
PCT-371-DATE: May 3, 1994
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PCT-PUB-NO: WO92/05447
PCT-PUB-DATE: April 2, 1992

INT-CL: [6] G01N 33/53, C12Q 1/68, C12P 21/02, C12N 15/09, C12N 5/10

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US-CL-CURRENT: 435/4; 435/375, 435/6, 435/7.1, 435/70.1

FIELD-OF-SEARCH: 435/4, 435/6, 435/7.1, 435/69.1, 435/70.1, 435/71.1, 435/172.3, 435/240.2

REF-CITED:

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ART-UNIT: 189

PRIMARY-EXAMINER: Chambers; Jasemine C.

ASSISTANT-EXAMINER: Priebe; Scott D.

ATTY-AGENT-FIRM: Gray Cary Ware & Freidenrich Reiter; Stephen E.

ABSTRACT:

Hormone receptors and the transcription factor Jun/AP-1 have been shown to reciprocally repress one another by a mechanism which is independent of DNA binding. For example, over-expression of AP-1 represses glucocorticoid-induced activation of genes carrying a functional glucocorticoid response element. Conversely, glucocorticoid has been shown to repress the transcriptional activation of genes which are controlled by promoters which contain the AP-1 binding site. In addition, methods are disclosed for selecting compounds useful for treating cells undergoing uncontrolled proliferation, such compounds being capable of disrupting the function of AP-1, but display substantially no ability to promote the transcriptional activation of hormone responsive genes.

22 Claims, 17 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 14

GOVT-INTEREST:

This invention was made with Government support under grant GM26444, awarded by the National Institute of Health. The Government has certain rights in the invention.

BRIEF SUMMARY:

This invention relates to steroid hormones, steroid hormone-like compounds, steroid hormone receptors, steroid hormone-like receptors, and related species. In a particular aspect, this invention relates to processes mediated by steroids and related hormones. In a further aspect, this invention relates to processes mediated by the proto-oncogenic protein complex, AP-1.

BACKGROUND OF THE INVENTION

Steroids and related hormones play an important role in regulating development, differentiation and homeostasis. The hormones exert their regulatory effects by binding to a superfamily of intracellular receptors, which are direct modulators of gene transcription. Mutational analyses of hormone receptors have identified functional domains responsible for transcriptional activation, nuclear localization, DNA binding, and hormone binding.

Hormone receptors can act to both activate transcription, and to repress expression of a variety of genes. It has been postulated that such repression is mediated by binding of the hormone receptor to DNA regulatory sequences, termed negative hormone response elements, thereby displacing transcriptional activators.

It would be desirable to be able to control the degree to which hormones, either directly or indirectly, activate transactivation and/or the degree to which hormones, either directly or indirectly, repress the expression of certain genes, for such purposes as the treatment of disease states, the development of treating agents with reduced incidence of side effects, and so forth.

The AP-1 protein complex is a member of a class of nuclear proteins encoded by proto-oncogenes that have been implicated in diverse aspects of cell growth, differentiation, and development. The AP-1 binding site is recognized by c-Jun homodimers and c-Jun/c-Fos heterodimers. Binding of c-Fos to the AP-1 site is dependent on the formation of heterodimers with c-Jun. Homodimer and heterodimer formation is mediated through non-covalent interactions facilitated by a structure termed the leucine zipper. In addition to imparting positive regulatory effects on several pathways, the AP-1 complex has also been shown to confer negative regulation on several genes.

Up until now, the effect of a given protein on gene regulation has generally been thought to be the result of interaction between the protein and a regulatory element within the promoter region of the gene being regulated. Thus, compounds which exert an effect on more than one pathway are thought to recognize a responsive element which is common to more than one pathway. Consistent with this, Diamond et al., [in Science 249: 1266-1272 (1990)] describe studies employing a "composite" glucocorticoid response element (GRE), which binds selectively in vitro to both glucocorticoid receptor and c-Jun and c-Fos (components of the phorbol ester-activated AP-1 transcription factor). The authors then propose a general model for composite GRE action that requires DNA binding for interaction between receptor (i.e., glucocorticoid receptor) and non-receptor factors (i.e., c-Jun or c-Fos).

Based on the above-described understanding of the mechanism by which regulatory proteins exert their effects, it would not be possible to alter one regulatory effect of a given protein without also altering some other regulatory effects of that protein. Thus, for any beneficial effect achieved by administration of a hormone or hormone analog, there is a strong likelihood that an undesirable side effect will occur, i.e., promotion of undesired processes and/or inhibition of desired processes. Accordingly, there has been no motivation in the art to search for compounds which are capable of disrupting a known pathway without also undesirably impacting other regulatory pathways.

SUMMARY OF THE INVENTION

In accordance with the present invention, we have discovered that hormone receptors and transcription factor, AP-1, can reciprocally repress each other's transcriptional activation activity. Similarly, we have discovered that hormone receptors and transcription factor, AP-1, can reciprocally derepress each other's ability to inhibit expression of certain genes. This is believed to occur via a novel mechanism which is independent of DNA binding.

The present invention, therefore, provides means to control the transcription activation of hormone-responsive gene products, and/or AP-1 responsive gene products. In addition, the present invention provides means to screen for compounds that inhibit cell growth, but which do not promote differentiation of said cells.

DRAWING DESCRIPTION:

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1A and FIG. 1B show the repression of glucocorticoid-mediated induction of

transcription by the transcription factor c-Jun.

FIG. 2A, FIG. 2B and FIG. 2C show the repression of the collagenase promoter by the glucocorticoid receptor.

FIG. 3A and FIG. 3B show the repression of an AP-1-induced collagenase promoter-CAT reporter by the glucocorticoid receptor.

FIG. 4 summarizes the results of a deletion study to determine domains of the glucocorticoid receptor which repress AP-1 induced expression and induce glucocorticoid-mediated transcription.

FIG. 5 presents a deletion analysis of the c-Jun gene, with an indication of the ability of the various deletion mutants to repress glucocorticoid-mediated transcription activation.

FIG. 6A, FIG. 6B and FIG. 6C present gel retardation assays performed to investigate the ability of c-Jun to repress the binding of the glucocorticoid receptor to a glucocorticoid response element.

FIG. 7 shows the repression of RAR-mediated repression of collagenase promoter activity.

FIG. 8 shows the repression of the collagenase promoter by the retinoic acid receptor-alpha.

FIG. 9 summarizes the results of a deletion study to determine domains of the retinoic acid receptor which repress AP-1 induced expression by the collagenase promoter.

FIG. 10 presents gel retardation assays performed to determine the ability of RAR to repress the binding of c-Jun to an AP-1 binding site.

FIG. 11 presents gel retardation assays performed to investigate the ability of RAR to repress the binding of AP-1 to an AP-1 binding site.

DETAILED DESCRIPTION:

DETAILED DESCRIPTION OF THE INVENTION

In accordance with the present invention, there is provided a method for identifying compound(s) useful for treating abnormal cells, said method comprising selecting a compound which displays both:

(a) the ability to disrupt the function of AP-1, when said compound is employed in a first assay system comprising a cell line capable of expressing:

(i) steroid hormone or steroid hormone-like receptor,

(ii) AP-1, and

(iii) AP-1-responsive reporter; and

(b) substantially no ability to promote the transcriptional activation of steroid hormone-responsive or steroid hormone-like responsive genes, when said compound is employed in a second assay system comprising a cell line capable of expressing:

(i) steroid hormone or steroid hormone-like receptor, and

(ii) steroid hormone- or steroid hormone-like responsive reporter.

In accordance with another embodiment of the present invention, there is provided a method for identifying compound(s) which disrupt the AP-1 response pathway, but which exert substantially no effect on steroid hormone or steroid hormone-like responsive pathways, said method comprising:

(a) testing said compound in a first assay system to determine the effect of said compound on the AP-1 responsive pathway; wherein said first assay system comprises a cell line capable of expressing:

(i) steroid hormone or steroid hormone-like receptor,

(ii) AP-1, and

(iii) AP-1-responsive reporter, and

(b) testing said compound in a second assay system to determine the effect of said compound on the transcriptional activation of steroid hormone or steroid hormone-like responsive genes; wherein said second assay system comprises a cell line capable of expressing:

(i) steroid hormone or steroid hormone-like receptor, and

(ii) steroid hormone-responsive or steroid hormone-like-responsive reporter; and thereafter

selecting those compounds which have an inhibitory effect in the testing of part (a), and substantially no effect in the testing of part (b).

In accordance with a further embodiment of the present invention, there is provided a method to repress, in an expression system, transcription activation of steroid hormone-responsive or steroid hormone-like-responsive gene(s) by steroid hormones or steroid hormone-like compound(s), said method comprising:

exposing said system to compound(s) and/or condition(s) which induce AP-1 expression, effective to repress expression of said steroid hormone-responsive or steroid hormone-like-responsive gene(s).

It is desirable that the expression systems employed in this embodiment of the present invention be responsive to steroid hormone or steroid hormone-like compounds, while at the same time being substantially non-responsive to the presence of c-Jun, c-Fos, or AP-1.

In accordance with yet another embodiment of the present invention, there is provided a method to repress, in an expression system, transcription activation of steroid hormone-responsive or steroid hormone-like-responsive gene(s) by steroid hormone or steroid hormone-like compound(s), or analogs thereof, said method comprising:

administering to said system a peptide comprising the leucine zipper region of c-Jun, or a functional analog thereof, in an amount effective to repress expression of said steroid hormone-responsive or steroid hormone-like-responsive gene(s).

As with the preceding embodiment of the present invention, it is desirable for the expression systems employed in this embodiment of the present invention to be responsive to steroid hormone or steroid hormone-like compounds, while being substantially non-responsive to the presence of c-Jun, c-Fos, or AP-1.

In accordance with still another embodiment of the present invention, there is provided a method to repress, in an expression system, transcription activation of AP-1-responsive gene(s) by AP-1, or analogs thereof, said method comprising:

administering to said system a composition comprising:

(i) functional ligand-binding domain of steroid hormone receptor or steroid hormone-like receptor, or analog thereof, and

(ii) functional DNA-binding domain of steroid or steroid-like receptor, or analogs thereof,

in an amount effective to repress expression of said AP-1-responsive gene(s).

It is desirable that the expression systems employed in this embodiment of the present invention be responsive to c-Jun, c-Fos, or AP-1, while being substantially non-responsive to the presence of steroid hormone or steroid hormone-like compounds.

In accordance with a still further embodiment of the present invention, there is provided a method to overcome, in the presence of asteroid hormone or steroid hormone-like compound, or analog thereof, the repression of expression of gene product(s) from genes subject to negative regulation by steroid hormone receptors, steroid hormone-like receptors or analogs thereof, said method comprising:

exposing said system to compound(s) and/or condition(s) which induce AP-1

expression, in an amount effective to suppress the repression of expression of said gene product(s).

In accordance with a further embodiment of the present invention, there is provided a method to overcome the inhibition of proliferation and function of lymphoid cells by asteroid hormone, steroid hormone-like compound, or analog thereof, in the presence of asteroid hormone receptor or steroid hormone-like receptor, or analog thereof, said method comprising:

exposing said system to compound(s) and/or condition(s) which induce AP-1 expression, effective to suppress the inhibition of proliferation and function of said lymphoid cells.

In accordance with a still further embodiment of the present invention, there is provided a compound which forms a first complex with asteroid hormone or steroid hormone-like receptor; wherein said first complex, in the presence of AP-1, disrupts the function of AP-1; and wherein said first complex is substantially unable to promote transcriptional activation of steroid hormone or steroid hormone-like responsive genes.

Hormone-mediated transcription activation has been elucidated for many hormones; and for some hormones, this mode of activation can effect many different genes. It is sometimes desirable to modulate this transcription activation. In accordance with the present invention, this can be accomplished by either exposing the system to compound(s) and/or condition(s) which induce AP-1 expression, or by administering to the system a peptide comprising the leucine zipper region of c-Jun, or analogs thereof, in an amount effective to repress expression of the hormone responsive gene product.

Compounds which are capable of inducing the expression of AP-1 include compounds which induce tumor formation (e.g., phorbol esters), growth factors (e.g., EGF, FGF, CSF), cytokines (e.g., IL-1, IL-2), neuropeptides (e.g., somatostatin), neurotransmitters (e.g., acetylcholine), protein kinase c (and compounds capable of inducing protein kinase c, e.g., EGF, insulin, platelet-derived growth factor, alpha-1 andronergic agents, IL-1, IL-2, and the like), and the like.

Conditions which are capable of inducing the expression of AP-1 include exposure of the system to ultraviolet irradiation, gamma irradiation, heat shock, stress, and the like.

Alternatively, instead of inducing the expression of endogenous (or exogenous) AP-1, the invention process can be accomplished by administering effective amounts of the c-Jun leucine zipper region to the system. Administration of a peptide comprising this component can be accomplished in a variety or ways, e.g., by direct introduction of purified or semi-purified peptide composition containing the desired component; by inducing expression of a gene construct encoding the leucine zipper region; and the like.

The leucine zipper region is a fragment of at least 29 amino acids, which orient themselves in an alpha-helix, wherein each seventh amino acid of the amino acid chain is a leucine, so that the leucine residues of one alpha-helix can interdigitate with the leucine residues of a second alpha-helix, e.g., another c-Jun moiety, (thereby producing homodimer), a c-Fos moiety (thereby producing heterodimer), and the like.

The molar ratio of protein comprising the c-Jun leucine zipper region, relative to the molar amount of steroid hormone receptor present in the expression system can vary widely. Broadly, ratios in the range of about 0.5 up to 100:1 are useful. Preferably, ratios of AP-1 component (or derivatives thereof) to steroid hormone receptor will fall in the range of about 1 up to 20:1; with molar ratios in the range of about 5 up to 15:1 being the presently most preferred ratio.

Steroid hormone or steroid hormone-like responsive genes contemplated for use in this embodiment of the present invention include glucocorticoid-responsive gene(s), retinoic acid-responsive gene(s), vitamin R-responsive gene(s), thyroid hormone responsive gene(s), mineralocorticoid-responsive gene(s), estrogen-responsive gene(s), estrogen-related hormone-responsive gene(s), androgen-responsive gene(s), progesterone-responsive gene(s), retinoid-responsive gene(s), arylhydrocarbon-responsive gene(s), and the like.

The invention method for the modulation of hormone induced transcription activation can be employed to treat a subject displaying a disease state. Disease states which are amenable to such treatment include anorexia nervosa, alcoholism, severe depression, chronic stress syndrome (which diseases are associated with

suppression of the immune system caused by abnormally high levels of glucocorticoids), and the like.

Hormones are also known to exert negative regulation on certain processes. It is sometimes desirable to modulate this negative regulation. In accordance with the present invention, this can be accomplished by either exposing the system to compound(s) and/or condition(s) which induce AP-1 expression, or by administering to said system a peptide comprising the leucine zipper region of c-Jun, or analogs thereof, in an amount effective to suppress the hormone-mediated repression of expression of gene products.

Genes subject to negative regulation by steroid hormones or steroid hormone-like compounds include the pro-opiomelanocortin gene, the prolactin gene, the proliferin gene, the chorionic gonadotropin alpha-subunit gene, the phosphoenolpyruvate carboxykinase gene, and/or the collagenase gene.

AP-1-mediated transcription activation has also been elucidated for numerous gene products. It is sometimes desirable to modulate this transcription activation. In accordance with the present invention, this can be accomplished by administering to the AP-1-responsive system a composition comprising:

(i) functional steroid hormone receptor or steroid hormone-like receptor ligand-binding domain, or analog thereof, and

(ii) functional steroid hormone receptor or steroid hormone-like receptor DNA-binding domain, or analog thereof, in an amount effective to repress expression of gene products.

The composition employed in this embodiment of the present invention can be administered as a single protein containing both the ligand-binding domain and the DNA binding domain, or as two separate proteins, each providing one of the desired functions. It is presently preferred, for ease of handling, that the two desired functions be provided as part of a single protein.

Regardless of whether the composition employed in this embodiment of the invention is administered as one or two protein species, the composition can be introduced into the system to be modulated in a variety of ways. For example, purified or semi-purified protein(s) can be administered directly to the system. Alternatively, expression vector(s) encoding the desired protein(s) can be induced to express such products.

The molar ratio of composition comprising the ligand binding domain and DNA binding domain, relative to AP-1 present in the expression system, can vary widely. Broadly, ratios in the range of about 0.5 up to 100:1 are useful. Preferably, ratios of composition to AP-1 will fall in the range of about 1 up to 20:1; with molar ratios in the range of about 5 up to 15:1 being the presently most preferred.

The method of the invention can be employed in a variety of ways, e.g., for treating disease states which are stimulated by AP-1. Such disease states include tumor formation (e.g., formation of lymphomas), arthritis, asthma, allergies, rashes, and the like.

Hormone receptors contemplated for use in the practice of the present invention include the intracellular steroid receptors, such as, for example, glucocorticoid receptor(s), retinoic acid receptor(s), vitamin D.sub.3 receptor(s), thyroid receptor(s), mineralocorticoid receptor(s), estrogen receptor(s), estrogen-related receptor(s), retinoid receptor(s), androgen receptor(s), progesterone receptor(s), arylhydrocarbon receptor(s) and the like. Presently preferred receptors include glucocorticoid receptor(s), thyroid receptor(s), mineralocorticoid receptor(s), estrogen receptor(s), estrogen-related receptor(s), retinoid receptor(s), androgen receptor(s) and progesterone receptor(s). The presently most preferred receptor for use in the practice of the present invention is the glucocorticoid receptor, because this receptor has been particularly thoroughly characterized.

In accordance with one embodiment of the present invention, a compound useful for treating abnormal cells can be identified by screening for compounds which meet the two criteria of disrupting the function of AP-1, but which fail to promote transcriptional activation of steroid hormone-responsive genes.

A convenient means to assess the ability of test compound to disrupt the function of AP-1 is to employ the test compound in an assay system comprising a cell line capable of expressing steroid hormone receptor, AP-1, and AP-1-responsive

reporter. Cells which express endogenous receptor, AP-1 and AP-1-responsive reporter, or cells having an exogenous source of one or more of the above can be employed. Preferred cells to employ for this purpose are cells which do not have a "hormone response element" associated with the AP-1 responsive reporter.

If the compound is effective in disrupting the function of the AP-1 pathway, the AP-1 responsive reporter (a gene product which can be readily measured by conventional methods) will not be expressed. Conversely, if the compound fails to disrupt the AP-1 responsive pathway, the AP-1 responsive reporter will be expressed and can readily be measured.

A convenient means to assess the ability of test compound to promote (or fail to promote) transcriptional activation of steroid hormone responsive genes, is to employ the test compound in an assay system comprising a cell line capable of expressing a hormone receptor and a hormone-responsive reporter. Cells expressing endogenous steroid hormone receptor and/or steroid hormone-responsive reporter can be employed. Alternatively, cells transfected with an exogenous source of steroid hormone receptor and/or steroid hormone-responsive reporter can be employed. If the test compound promotes transcriptional activation, the steroid hormone-responsive reporter will be expressed, and can readily be measured. Conversely, if the test compound does not promote transcriptional activation, the steroid hormone-responsive reporter will not be expressed.

The invention will now be described in greater detail by reference to the following non-limiting examples.

EXAMPLES

Experimental procedures employed in the examples described herein are set forth below:

Recombinant Plasmids

The collagenase-CAT constructs and plasmid (AP-1).sub.5 -TKCAT have been described by Angel et al., (See Mol. Cell. Biol., 1: 2256-2266 (1987).

The recombinant GR mutants have been described by Hollenberg et al., (See Cell, 49: 39-46 (1987) and Cell 55: 899-906 (1988)), Giguere et al., Nature 330: 624-629 (1987), and Umosono and Evans Cell 57: 1139-1146 (1989).

The RAR mutant RAR.alpha..DELTA.1-80 was generated by replacing the NotI-BamHI fragment of GR13 with that of RAR.sub.NX [see Hollenberg and Evans in Cell 86: 899-906 (1988)]. Mutant RAR.alpha..DELTA..sub.NX was generated by replacing the NotI-XhoI fragment of RAR.alpha..sub.NX with a synthetic oligonucleotide containing NotI-XhoI restriction sites.

The recombinant mouse c-Jun constructs have described by Lamph et al., Nature 334: 629-631 (1988) and Ransone et al., (See Genes and Devel. 3: 770-781 (1989) and Proc. Nat'l Acad. Sci. U.S.A. 87: 3806-3810 (1990)). Construct SVTLZJun was created by amplification of DNA sequences coding for amino acids 282 to 334 of mouse c-Jun (Lamph et al., supra) by polymerase chain reaction and subsequent ligation downstream of a SV40 nuclear translocation signal (Kalderon et al., Cell 39: 499-509 (1984) in a pRS expression vector (Giguere et al., Cell 46: 645-652 (1986). The sequence was confirmed according to Taboe and Richardson, Proc. Nat'l. Acad. Sci. U.S.A. 84: 4767-4771 (1987).

Transfections and Reporter Assays

Transfection of plasmid DNA into HeLa, NIH3T3, or CV-1 cells was performed using the standard calcium phosphate co-precipitation technique described by Gorman et al., in Mol. Cell. Biol. 2: 1044-1051 (1982) with minor modifications described by Schule et al., in Nature 332: 87-90 (1988). Cells were maintained in DMEM medium supplemented with 10% bovine calf serum (BCS). Twenty-four hours before transfection, 7.times.10.sup.5 cells/100 mm dish were plated in phenol-red free DMEM supplemented with 10% charcoal-treated bovine calf serum (BCS). Typically 2 .mu.g of reporter plasmid and 2 .mu.g of an RAS-.beta.-galactosidase (internal control for transfection efficiently) expression plasmids (Umesono and Evans, supra) were used. Co-transfection of additional expression constructs is indicated in each example. The total amount of transfected DNA was always adjusted to 20 .mu.g with pUC18. The cells were exposed to the precipitate for 16-20 hours. Unless otherwise indicated, the cells were refed with phenol-red free DMEM, 10% charcoal-treated BCS and 10.sup.-7 M DEX (or 10.sup.-6 M RA) was added. For TPA induction of Col-CAT reporter constructs, HeLa cells were refed with phenol-red free DMEM supplemented with 0.5% charcoal-treated BCS and

incubated simultaneously with 100 ng/ml TPA and 10.sup.-7 M DEX.

Protein-DNA Binding Assays

Three .mu.l of freshly in vitro-translated proteins were pre-incubated in binding buffer [10 mM HEPES, pH 7.8/4 mM MgCl.sub.2 /0.1 mM EDTA/4 mM spermidine/2 mM dithiothreitol, bovine serum albumin at 100 .mu.g/ml/poly(dI-dC) at 1 .mu.g/ml/15% (vol/vol) glycerol] on ice for ten minutes. Subsequently, 2 ng of .sup.32 P-labeled oligonucleotide probes (4.times.10.sup.4 dpm) was added to the reaction mixture. For the competition assay, various amounts of bacterially expressed GR, RAR.alpha. or untransformed bacterial BL-21 lysate were added simultaneously with Jun proteins. Jun proteins were either translated in vitro or obtained from HeLa cell extracts. After additional incubation on ice for 15 minutes, the protein-DNA complexes were resolved by 4% PAGE in 45 mM Tris/32.3 mM boric acid/1.25 mM EDTA, pH 8.3. The dried gel was then exposed with intensifying screen at -70.degree. C. with Kodak XAR film.

EXAMPLE 1

Jun Represses GR Mediated Activation

c-Jun and GR expression plasmids were co-transfected into NIH3T3 cells and assayed to see whether c-Jun was able to inhibit GR-mediated activation of a GRE.sub.2 -TKCAT reporter plasmid [Schule et al., Science 242: 1418-1420 (1988)]. NIH3T3 cells were used in this experiment because they contain endogenous GR and upon starvation express only residual amounts of the AP-1 complex. As shown in FIG. 1, GR strongly induced reporter activity upon the addition of the synthetic glucocorticoid dexamethasone.

Co-transfection was carried out with constant amounts of GR plasmid expression and various amounts of c-Jun expression plasmid, .DELTA.Jun, a construct lacking the c-Jun coding sequences and .DELTA.RKJun, a construct lacking the c-Jun DNA binding domain, were also included in the Experiment. In FIG. 1, the cells are shown as either untreated (black bars) or treated with DEX (striped bars). Numbers presented in the Figure indicate g of co-transfected plasmid DNA. As shown in FIG. 1A, GR strongly induced reporter activity upon the addition of the synthetic glucocorticoid dexamethasone (DEX) (FIG. 1A, lane 1). Co-transfection of increasing amounts of c-Jun expression plasmids inhibited hormone-induced reporter activity in a concentration-dependent manner (FIG. 1A, compare lanes 1 with 2-5). In contrast, transfection of parental plasmid .DELTA.Jun, which lacks c-Jun coding sequences, did not alter the activity of GRE.sub.2 -TKCAT (FIG. 1A, lane 7). Because the GRE.sub.2 -TKCAT reporter lacks an intrinsic AP-1 site, inhibition does not appear to require binding of c-Jun to DNA. This was corroborated by a mutant, .DELTA.RKJun lacking a functional DNA binding domain, (FIG. 1A, lane 6). This mutant produced levels of repression similar to that of the wild-type c-Jun protein (FIG. 1A, compare lanes 5 and 6). The expression of control plasmid TKCAT, lacking the GRE, was not influenced by either hormone treatment or over-expression of c-Jun. c-Jun also repressed the hormone dependent activation of GRE.sub.2 -TKCAT by endogenous GR present in NIH3T3 cells (FIG. 1B, compare lanes 1 AND 2).

To further demonstrate that c-Jun mediated repression is not specific for a particular GRE or promoter sequence, we transfected .DELTA.MGRE.sub.p -CAT reporter plasmid (Thompson and Evans, Proc. Nat'l. Acad. Sci. U.S.A. 86: 3494-3498 (1989) containing a palindromic GRE in the mouse mammary tumor virus promoter was transfected into NIH3T3 cells. Transcriptional activity of GRE.sub.2 -TKCAT, .DELTA.MGRE.sub.p -CAT and the control plasmid AM-CAT in NIH3T3 cells in the absence (black bars) or presence (striped bars) of DEX is shown in FIG. 1B. Transcriptional activity was analyzed with endogenous receptor activity present in NIH3T3 cells (lanes 1-2) or in cells transfected with 0.5 .mu.g of GR expression plasmids (lanes 3-6). Where indicated (+)5 .mu.g of C-Jun expression plasmids were co-transfected. The NIH3T3 cells were cultured in low (0.5%) serum. The activity of this construct was efficiently induced by GR in the presence of hormone (FIG. 1B, lane 3) and repressed by over-expression of c-Jun (FIG. 1B, lane 4). Neither hormone treatment nor over-expression of c-Jun significantly altered activity of the control plasmid .DELTA.M-CAT (FIG. 1B, lanes 5 and 6).

EXAMPLE 2

The Collagenase AP-1 Site Is Required for DEX Repression

In this example, the ability of the GR to inhibit induction of an AP-1 responsive promoter was examined. The AP-1 inducible reporter construct (AP-1).sub.5 -TKCAT was transfected into GR negative CV-1 cells cultured in low (0.5%) serum (FIG.

2A). FIG. 2A summarizes the activity of reporter construct (AP-1).sub.5 -TKCAT and the control plasmid TKCAT in untreated (shown as black bars) or DEX treated (shown as striped bars) CV-1 cells co-transfected with either GR expression plasmid or parental vector .DELTA.GR lacking the GR coding sequences, and Jun/Fos expression plasmids. The CV-1 cells were cultured in low (0.5%) serum. This promoter has a high basal activity which is further stimulated (FIG. 2A, lane 2) by the cotransfection of Jun/Fos expression vectors. The presence of DEX and GR leads to potent inhibition of this induction (lane 2), whereas the control plasmid .DELTA.GR, lacking the GR coding sequences, has no effect on Jun/Fos induction in the presence of DEX (lane 3). As shown with the TKCAT control (lanes 4 and 5), induction is dependent on the presence of the AP-1 sites and, in the absence of these sites, glucocorticoids alone have no effect.

The collagenase promoter provided an opportunity to examine this potential regulation of a cellular gene by GR. This gene was chosen because glucocorticoids have been shown to negatively regulate its expression [See Brinckerhoff et al., Biochemistry 25: 6378-6384 (1988)]. Whereas Jun/AP-1 and factors stimulating the Jun/AP-1 pathway are known to positively induce its activity, various collagenase promoter-CAT reporter plasmids were transfected into HeLa or CV-1 cells, respectively. HeLa and CV-1 cells were used because collagenase is expressed in both cell lines [Angel et al., Mol. Cell. Biol. 7: 2256-2266 (1987)]. In addition, HeLa cells express endogenous GR activity. FIG. 2B shows transcriptional activity of various collagenase promoter-CAT deletion mutants and heterologous reporters in HeLa cells cultured in 10% serum in the absence (black bar) or presence of DEX (striped bar). In the Figure, "TK" refers to the thymidine kinase-CAT construct; "(AP-1).sub.5 TK" refers to a construct comprising five copies of the collagenase AP-1 site in front of TK-CAT; and ".DELTA.MGRE.sub.p " refers to a construct comprising a consensus GRE cloned into DMCAT. As shown in FIG. 2B (lanes 1-3) addition of DEX to HeLa cells resulted in a 4-5 fold repression of 1200 Col-CAT and 73 Col-CAT reporter activity respectively, whereas the activity of the reporter plasmid 63 Col-CAT remained unchanged. These results indicate that repression is mediated by DNA sequences located between position -73 and -63 in the collagenase promoter. This region has been shown to contain a TPA-inducible enhancer which is recognized by AP-1 complex. The DEX responsive reporter .DELTA.MGRE.sub.p -CAT was activated in a hormone-dependent manner, indicating that the repression of the collagenase promoter or a heterologous reporter is dependent on the presence of the AP-1 site (FIG. 2B, lanes 6).

To demonstrate that repression is not cell-type specific, GR expression vector was co-transfected together with the Col-CAT reporter plasmids into GR-negative CV-1 cells. FIG. 2C shows transcriptional activity of collagenase promoter deletion mutants and .DELTA.MGRE.sub.p -CAT in CV-1 cells cultured in 10% serum and co-transfected with either GR expression plasmid or the control plasmid (.DELTA.GR) in the absence (black bar), or presence of DEX (striped bar). Lane 1 of FIG. 2C shows that addition of DEX to CV-1 cells transfected with parental plasmids lacking the coding sequence for GR did not alter Col-CAT activity. In contrast, in the presence of the receptor, 1200 Col-CAT and 73 Col-Cat were potently repressed in a hormone dependent manner (FIG. 2C, lanes 2-3) while 63 Col-CAT was only slightly affected by the activated GR (FIG. 2C, lane 4).

To further explore the interaction between GR and Jun/AP-1, Col-CAT reporter activity was increased by either treatment with TPA or, alternatively, by over-expressing the Jun and Fos proteins. Expression of Collagenase-CAT deletion mutants was measured in untreated HeLa cells incubated with either TPA, DEX, or a combination of both. Transcriptional activity of Collagenase-CAT deletion mutants was measured in untreated (black bars) or DEX-treated (striped bars) CV-1 cells co-transfected with either GR expression plasmid, parental vector .DELTA.GR lacking the GR coding sequences, or Jun/Fos expression plasmids. Both HeLa and CV-1 cells were cultured in low (0.5%) serum. As shown in FIG. 3A, addition of TPA to HeLa cells cultured in low (0.5%) serum strongly elevates 1200 Col-CAT activity (compare lanes 1 and 3). This induction was severely reduced by the stimulation of endogenous GR receptors by DEX addition (FIG. 3A, lane 4). Expression of 63 Col-CAT was only weakly affected by either treatment (FIG. 3A, lanes 5-8). Expression of 1200 Col-CAT can be elevated by co-transfecting Jun/Fos expression plasmids into CV-1 cells cultured in low (0.5%) serum (FIG. 3B, lane 2). These induced levels were efficiently repressed by DEX-activated GR (FIG. 3B, compare lanes 1 and 2). No repression was seen when the parental plasmid .DELTA.GR lacking the GR coding sequences, was used (FIG. 3B, lane 3). Neither hormone treatment nor over-expression of Jun/Fos altered activity of the control plasmid 63 Col-CAT significantly (FIG. 3B, lanes 4 and 5).

The data shown in FIGS. 2 and 3 demonstrate that both activation and repression of the collagenase promoter and the TK reporter is dependent on the presence of

transcriptional activity of the AP-1 site. The AP-1 site is the major enhancer in the promoter of the collagenase gene and the only enhancer of (AP-.sub.1).sub.5 -TKCAT. Thus, glucocorticoids may function as general modulators of AP-1 responsive genes.

EXAMPLE 3

GR DNA Binding Domain Is Necessary But Not Sufficient for Repression of Jun/AP-1 Activity

The above experiments demonstrate that Jun/AP-1 activation can be efficiently repressed by GR in a hormone dependent manner. To define regions of the receptor involved in repression, several GR mutants were analyzed in co-transfection studies in CV-1 cells for their ability to repress 1200 Col-CAT reporter activity. In FIG. 4, GR mutants previously characterized for transcriptional activation, nuclear localization, DNA binding, and ligand binding were assayed for their ability to repress activity of the 1200 Col-CAT reporter or to activate the MTV-CAT reporter. The wild-type receptor consists of two activation domains (.tau.1 and .tau.2), the DNA binding domain (DNA) and a ligand binding domain (ligand). In FIG. 4, the scale above the depicted mutants indicates amino acid position numbers. The deleted amino acids are indicated on the left. In some mutants, the GR DNA binding domain is replaced by the DNA binding domains of GAL4 (GgalG), TR (GTG), or RAR (GRG). The recombinant mutant RGR is composed of the GR DNA binding domain plus RAR amino- and carboxy-termini. The mutant GTG3A contains three point mutations (EG--G) in the P-box of the GR DNA binding domain. Repression of 1200 Col-CAT reporter activity obtained with wild-type GR was set at 100%. All mutants were expressed in similar amounts in the cell as assayed by Western blot analysis. The hybrid receptors shown in rows 13-17 activate their cognate response elements in a hormone-dependent manner. Most amino-terminal deletion mutants exhibited reduced glucocorticoid-mediated repression (FIG. 4, compare lane 1 with lanes 2-5). Interestingly mutant .DELTA.240-290 (lane 3), which has a short deletion in a transcriptional activation domain, termed .tau..sub.1, repressed reporter activity better than wild-type GR. The carboxy-terminal truncation mutants 550* and 515* both exhibited markedly reduced hormone independent repression activity (lane 6 and 8). These data show that the ligand binding domain and, to a lesser extent, the amino terminus contribute to repressor activity.

Deletion of the entire DNA binding domain resulted in a complete loss of repression (lane 10, mutant .DELTA.428-490). Further analysis revealed that deletion of either the first zinc finger (lane 11, mutant .DELTA.420-451) or the second zinc finger (lane 12, mutant .DELTA.450-487) of the GR completely eliminated repression. Although the DNA binding domain is necessary for repression, it is not sufficient, since mutants expressing only this region (lane 7, mutant .DELTA.9-385/550* and lane 9, mutant .DELTA.9-385/515*) are also inactive. Substitution of the GR DNA binding domain for that of the yeast transcription factor GAL4 resulted in a mutant which failed to repress (lane 13, mutant GgalG), even though it is able to activate GAL4 responsive promoters in a hormone-dependent fashion. The importance of the GR DNA binding domain is unexpected because GR does not bind to the collagenase AP-1 site (as shown in FIG. 6).

To provide further evidence that DNA binding is not necessary for GR-mediated repression (in contrast to the requirement for DNA binding in order for the interaction observed by Diamond et al., supra, to occur), a mutant receptor having a changed target gene specificity was examined to ascertain if it had lost the ability to repress. This GR mutant, which was generated through point mutations in the P-box of the DNA binding domain, recognizes TRE or ERE instead of the GRE. This mutant is still able to repress with only slightly reduced efficiency (FIG. 4, lane 14). In similar experiments, mutants in which the GR DNA binding domain had been swapped with those of either the RAR or TR (mutants GRG and GTG), were capable of repressing 1200 Col-CAT expression (FIG. 4A, lanes 15 and 16). Finally, a mutant (RGR) in which the GR amino- and carboxy-termini had been exchanged with that of RAR also repressed efficiently (lane 17).

Together these results indicate that the GR DNA binding domain and an intact finger structure are required for efficient repression. However, the target gene specificity of the DNA binding domain does not appear to be important, as substitution of the DNA binding domains of several steroid hormone receptors for that of the GR did not abolish the repression activity. In addition, a mutant receptor which consists of RAR amino- and carboxy termini and the GR DNA binding domain still retained repression activity.

EXAMPLE 4

The c-Jun Leucine Zipper Is Required for Repression of GR Activity

To determine which region of the c-Jun protein is responsible for repression of GR-mediated activation, a series of mutant c-Jun proteins were tested in NIH3T3 cells cultured in low (0.5%) serum for their ability to repress GRE.sub.2 -TKCAT activity. GRE.sub.2 -TKCAT or TKCAT reporter constructs were co-transfected into NIH3T3 cells with 0.5 .mu.g GR expression plasmid with or without 5 .mu.g of one of the indicated mutants. The cells were cultured in low (0.5%) serum.

FIG. 5 shows reporter activity as fold induction. The c-Jun protein is depicted as consisting of an amino terminus (NH.sub.2), a DNA binding region (BR), a leucine zipper (LZ), and a carboxy terminus (C). The numbers above the depicted mutants indicate positions of amino acids at the start or end of the corresponding region. The mutant SVTLZJun contains an SV40 nuclear translocation signal cloned in front of the leucine zipper. The c-Jun mutants were expressed in similar amounts. Deletion of either the c-Jun DNA binding domain (FIG. 5, lane 3) or the entire amino terminus (FIG. 5, lane 5) did not alter the ability of the c-Jun protein to repress GRE.sub.2 -TKCAT reporter activity. Deletion of the leucine zipper, however, abolished the protein's ability to repress (FIG. 5, lane 4).

To further examine the ability of the c-Jun leucine zipper to repress GR-mediated activation, a mutant was constructed which fused the leucine zipper plus 23 carboxy-terminal amino acids to a SV40 nuclear localization signal. As shown in FIG. 5, lane 6, this mutant repressed reporter activity at levels similar to wild-type c-Jun. Activity of control plasmid TKCAT was not altered by either hormone treatment or over-expression of c-Jun (FIG. 5, lanes 7 and 8).

The results shown in FIGS. 4 and 5 demonstrate that c-Jun is able to efficiently repress GR-mediated activation and that the carboxy-terminal region of c-Jun containing the leucine zipper is sufficient for this effect.

EXAMPLE 5

Inhibits GR-GRE Complex Formation

To test for a potential physical interaction between c-Jun and GR, gel retardation assays were performed. These assays were performed with .sup.32 P-labeled oligonucleotide containing a palindromic GRE and extracts prepared from COS cells transfected with constructs expressing either GR (FIG. 6A, lanes 1, 3, and 4) or beta-galactosidase (FIG. 6A, lane 2). Competition reactions were performed using a 50-fold excess of unlabeled oligonucleotide containing the GRE.sub.p (FIG. 6A, lane 3) or the collagenase AP-1 binding site (FIG. 6A, lane 4).

GR (obtained by over-expression in COS cells) formed a specific, retarded complex with an oligonucleotide containing a palindromic GRE (FIG. 6A, lane 1). The GR-GRE complex, while efficiently competed by a 50-fold excess of unlabeled GRE (lane 3), was unaffected by competition with a 50-fold excess of an oligonucleotide containing the AP-1 site found in the collagenase promoter (lane 4). This result indicates that the GR does not bind to the collagenase AP-1 binding site.

Gel retardation assays were performed with .sup.32 P-labeled oligonucleotides containing a palindromic GRE (FIG. 6B, lanes 1-8) or an NF-1 binding site (FIG. 6B, lanes 9-12) and extracts prepared from COS cells expressing the GR. Reactions were done in the absence (FIG. 6B, lanes 1, 5, and 9) or in the presence of 63 ng (FIG. 6B, lanes 2, 6, and 10), 130 ng (FIG. 6B, lanes 3, 7, and 11), or 250 ng (FIG. 6B, lanes 4, 8, and 12) of purified, bacterially-expressed c-Jun (FIG. 6B, lanes 1-4 and 9-12) or BL21 bacterial lysates (Mock) lanes 5-8).

Addition of increasing amounts of purified, bacterially expressed c-Jun is seen to result in a severe reduction in the amount of GR-GRE complex formed (FIG. 6B, lanes 2-4). In contrast, addition of mock-transformed bacterial lysate did not affect GR-GRE complex formation (FIG. 6B, lanes 5-8).

To demonstrate the specificity of the effect of c-Jun on GR-GRE interactions, gel retardation assays were performed using COS cell extracts (containing GR) and an oligonucleotide containing an NF-1 binding site. Addition of increasing amounts of c-Jun had no effect on NF-1 binding activity (FIG. 6B, lanes 9-12).

To further correlate these in vitro data with the in vivo results, a c-Jun truncation mutant, termed .DELTA.NJun, (see FIG. 5, lane 5) was assayed for its

ability to disrupt GR-GRE interactions. .DELTA.NJun, which has amino acids 1-249 deleted, retains the leucine zipper and basic domains of the c-Jun protein. Consistent with in vivo results, the truncation mutant was capable of disrupting formation of the GR-GRE complex (FIG. 6C; wherein gel retardation assays were performed with a .sup.32 P-labeled oligonucleotide containing a palindromic GRE and extracts prepared from COS cells which express the GR). Reactions were done in the absence (lanes 1 and 5) or presence of 200 ng (lanes 2 and 6), 400 ng (lanes 3 and 7), or 800 ng (lanes 4 and 8) of purified, bacterially-expressed mutant .DELTA.NJun (lanes 1-4) or BL21 bacterial lysates (Mock; lanes 5-8).

EXAMPLE 6

RAR Mediated Repression of Collagenase Promoter Activity

A reporter plasmid containing a 1.2-kilobase portion of the collagenase promoter fused to the bacterial chloamphenicol acetyltransferase (CAT) gene (1200Col-CAT) was co-transfected into HeLa cells along with 0.1 .mu.g of expression plasmids coding for RAR.alpha., RAR.beta. and RAR.gamma. (FIG. 7, bars 2-4), cultured in 10% serum and assayed to see whether the reporter gene is influenced by retinoic acid (RA). HeLa cells were used in this experiment because they contain endogenous RAR and express the collagenase gene as well as Jun protein. As shown in FIG. 7, endogenous RAR repressed 1200Col-CAT activity by about 50% upon RA addition (FIG. 7, bars 1). Co-transfection of any one of RAR.alpha., RAR.beta. or RAR.gamma. expression plasmids further inhibited 1200Col-CAT reporter activity to about 15% of the non-RA treated controls (FIG. 7, bars 2-4). In contrast, the control plasmid .beta.RE-TKCAT (which contains a known RA-response element, .beta.RARE [see Sucov et al., in Proc. Natl. Acad. Sci. USA 87: 5392-5396 (1990)]) was induced by RA, indicating that RA-mediated repression is specific for the collagenase promoter (FIG. 7, bars 5).

EXAMPLE 7

The AP-1 Site in the Collagenase Promoter Is Required for RA-Mediated Repression

In this example, those DNA sequences in the collagenase promoter (i.e., an AP-1 responsive promoter) that mediate repression by RA were examined. Thus, various collagenase-CAT reporter plasmids together with RAR.alpha. expression vectors were co-transfected into HeLa cells cultured in 10% serum in the absence (FIG. 8, black bars) or presence of RA (FIG. 8, striped bars).

FIG. 8 (bars 1-3) shows that addition of RA to HeLa cells resulted in about 6-fold repression of both 1200Col-CAT and 73Col-CAT reporter activity, whereas the activity of reporter plasmid 63Col-CAT remained unchanged.

These results indicate that repression is mediated by DNA sequences located between position -73 and -63 in the collagenase promoter. Thus, RAR, similar to GR, can inhibit induction of an AP-1 responsive promoter.

To further test the ability of RAR to inhibit induction of an AP-1 responsive promoter, the AP-1 inducible reporter construct (AP-1).sub.5 -TKCAT was transfected into HeLa cells. The high basal activity of this promoter is also repressed in the presence of RA and RAR.alpha. (FIG. 8, bar 4), whereas expression of the control TK promoter is not influenced by RA (FIG. 8, bars 5). As one might expect, the RA-responsive reporter .beta.RE-TKCAT was activated in a hormone dependent manner (FIG. 8, bars 6).

The data shown in FIG. 8 demonstrate that repression of the collagenase promoter or heterologous reporter by RA depends on the presence of the AP-1 site.

EXAMPLE 8

RAR DNA Binding Domain and a Region Near the C-Terminus Are Necessary but Not Sufficient for Repression of Jun/AP-1 Activity

The above experiments demonstrate that Jun/AP-1 activation can be efficiently repressed by RAR.alpha. in a hormone dependent manner. To define regions of the receptor involved in repression, several RAR mutants were analyzed in co-transfection studies in CV-1 cells for their ability to repress (AP-1).sub.5 -TKCAT reporter activity. In FIG. 9, the scale above each receptor indicates amino acid numbers. The wild type RAR.alpha. consists of the N terminus (amino acids 1-80), the DNA binding domain (amino acids 81-153), and the ligand binding domain (amino acids 154-462). The deleted amino acids are indicated at left. RA dependent repression of 1200Col-CAT reporter activity obtained by co-transfection with 0.1 .mu.g of RAR.alpha. expression plasmids was set at 100%.

Deletion of the entire N terminus of RAR did not impair the ability of the receptor to repress CAT activity (FIG. 9, compare bar 1 with bar 2). Deletion of the DNA-binding domain, however, resulted in a complete loss of repression (FIG. 9, bar 3).

Further analysis revealed that a mutant in which the RAR.alpha. DNA-binding domain had been swapped with that of the GR (RGR) was still able to fully repress (FIG. 9, bar 4). These results indicate that asteroid receptor DNA-binding domain is required for efficient repression. However, the target gene specificity of the DNA-binding domain is relatively unimportant.

Still further analyses revealed that both C-terminal truncation mutants 403* and 203* have completely lost the ability to repress (FIG. 9, bars 5 and 6). As another means to demonstrate the importance of the receptor ligand-binding domain, the C-terminus of RAR was exchanged with that of the oncogene v-erbA. The resulting mutant RRerbA also failed to repress (FIG. 9, bar 7).

EXAMPLE 9

Interferes with AP-1 Binding Activity

To test for a potential physical interactions between RAR and AP-1, gel retardation assays were performed. Bacterially expressed RAR.alpha. was unable to form a retarded complex with a .sup.32 P-labeled oligonucleotide containing the collagenase AP-1 site (FIG. 10, lane 2), indicating that RAR does not inhibit collagenase expression by directly binding to this sequence. In contrast, in vitro translated c-Jun formed a specific, retarded complex (FIG. 10, lane 3). Addition of increased amounts of bacterially expressed RAR.alpha. severely reduced the amount of complex formed in a dose-dependent fashion (FIG. 10, lanes 4-7), whereas mock-transformed BL21 bacterial lysate did not affect binding of c-Jun to DNA (FIG. 10, lanes 8-11).

Bacterially expressed RAR.alpha. also inhibited AP-1 DNA binding when HeLa cell extract was used as AP-1 source (See FIG. 11, comparing lanes 1 with 2-4). Addition of mock-transformed BL21 bacterial lysate did not affect complex formation (FIG. 11, lanes 5-8). As a control, gel retardation assays were performed using NF-1 activity present in HeLa cell extracts and an oligonucleotide containing an NF-1 binding site. Addition of increasing amounts of bacterially expressed RAR.alpha. had no effect on NF-1 binding activity, demonstrating the specificity of the inhibitory effect or RAR.alpha. on AP-1 DNA binding.

The invention has been described in detail with particular reference to preferred embodiments thereof, but it will be understood that variations and modifications can be effected within the spirit and scope of the invention.

CLAIMS:

That which is claimed is:

1. A method for selecting a compound, said method comprising selecting a compound which disrupts the function of Activator Protein-1 (AP-1), as determined by a first assay system, but does not promote transcriptional activation of a steroid hormone responsive gene, as determined by a second assay system, wherein:

(a) said first assay system comprises a suitable growth medium and a cell line that expresses, in said suitable growth medium:

(i) asteroid hormone receptor,

(ii) AP-1, and

(iii) an AP-1-responsive reporter;

(b) said determination by said first assay system comprises identifying a test compound which decreases expression of said AP-1-responsive reporter when said test compound is incubated in said first assay system, thereby identifying a compound which disrupts the function of AP-1;

(c) said second assay system comprises a second suitable growth medium and a cell line that expresses, in said second suitable growth medium:

(i) asteroid hormone receptor,

(ii) asteroid hormone-responsive reporter; and

(d) said determination by said second assay system comprises identifying a test compound which does mot increase expression of said steroid hormone-responsive reporter when said test compound is incubated in said second assay system, thereby identifying a compound which does not promote transcriptional activation of asteroid hormone-responsive gene.

2. The method according to claim 1 wherein said compound forms a first complex with asteroid hormone receptor; wherein said first complex, in the presence of AP-1, disrupts the function of AP-1; and wherein said first complex is substantially unable to promote transcriptional activation of steroid hormone responsive genes.

3. The method according to claim 1 wherein said receptor is a glucocorticoid receptor, a retinoic acid receptor, a vitamin D.sub.3 receptor, a thyroid receptor, a mineralocorticoid receptor, an estrogen receptor, an estrogen-related receptor, a retinoid receptor, an androgen receptor, or a progesterone receptor.

4. A method for identifying a compound which disrupts an Activator Protein-1 (AP-1) response pathway, but which exerts no substantial effect on asteroid hormone response pathway, said method comprising identifying a compound which has both an inhibitory effect on AP-1-responsive expression, as determined by a first assay system, and no substantial effect on steroid hormone-responsive expression, as determined by a second assay system, wherein:

(a) said first assay system comprises a suitable growth medium and a cell line that expresses, in said suitable growth medium:

(i) asteroid hormone receptor,

(ii) AP-1, and

(iii) an AP-1-responsive reporter;

(b) said determination by said first assay system comprises identifying a test compound which decreases expression of said AP-1-responsive reporter when said test compound is incubated in said first assay system, thereby identifying a compound which inhibits AP-1-responsive expression;

(c) said second assay system comprises a second suitable growth medium and a cell line that expresses, in said second suitable growth medium:

(i) asteroid hormone receptor,

(ii) asteroid hormone-responsive reporter; and

(d) said determination by said second assay system comprises identifying a test compound which does not increase expression of said steroid hormone-responsive reporter when said test compound is incubated in said second assay system, thereby identifying a compound which has no substantial effect on steroid hormone-responsive expression.

5. The method according to claim 4 wherein said receptor is a glucocorticoid receptor, a retinoic acid receptor, a vitamin D.sub.3 receptor, a thyroid receptor, a mineralocorticoid receptor, an estrogen receptor, an estrogen-related receptor, a retinoid receptor, an androgen receptor or a progesterone receptor.

6. A method to repress transcription activation of a steroid hormone-responsive gene by steroid hormones in an expression system that expresses a steroid hormone-responsive gene, said method comprising:

exposing said system to compounds or conditions which induce Activator Protein-1 (AP-1) expression, wherein said AP-1 expression represses expression of said steroid hormone-responsive gene.

7. The method according to claim 6 wherein said steroid hormone-responsive gene is glucocorticoid-responsive, retinoic acid-responsive, vitamin D.sub.3 -responsive, thyroid hormone responsive, mineralocorticoid responsive, estrogen responsive, estrogen-related hormone responsive, androgen-responsive, progesterone-responsive, or retinoid-responsive.

8. The method according to claim 6 wherein said steroid hormone-responsive gene is glucocorticoid-responsive, thyroid hormone responsive, mineralocorticoid responsive, estrogen responsive, estrogen-related hormone responsive, androgen-responsive,.progesterone-responsive, or retinoid-responsive.

9. The method according to claim 6 wherein said compounds or conditions which induce expression of AP-1 are compounds which induce tumor formation, growth factors, cytokines, neuropeptides, neurotransmitters, protein kinase c, or compounds which induce protein kinase c; or conditions of ultraviolet irradiation, gamma irradiation, stress or heat shock.

10. A method to repress transcription activation of a steroid hormone-responsive gene by steroid hormone compounds in an expression system that expresses a steroid hormone-responsive gene, said method comprising:

administering to said system a peptide comprising the leucine zipper region of c-Jun in an amount effective to repress expression of said steroid hormone-responsive gene.

11. The method according to claim 10 wherein the molar ratio of the leucine zipper region of c-Jun to steroid hormone receptor falls in the range of about 0.5:1 up to 100:1.

12. A method to repress transcription activation of an Activator Protein-1 (AP-1)-responsive gene by AP-1 in an expression system that expresses an AP-1-responsive gene, said method comprising:

administering to said system:

(a) a composition comprising:

(i) functional ligand-binding domain of steroid hormone receptor, and

(ii) functional DNA-binding domain of steroid hormone receptor, and

(b) a compound that binds to said ligand binding domain,

in an amount effective to repress expression of said AP-1-responsive gene.

13. The method according to claim 12 wherein said AP-1-responsive gene is a collagenase gene, a c-Jun gene, a c-Fos gene, an immune-response gene, or a retinoic acid receptor-alpha gene.

14. The method according to claim 12 wherein said composition comprises functional domains of asteroid hormone receptor selected from the group consisting of a glucocorticoid receptor, a retinoic acid receptor, a vitamin D.sub.3 receptor, a thyroid receptor, a mineralocorticoid receptor, an estrogen receptor, an estrogen-related receptor, a retinoid receptor, an androgen receptor and a progesterone receptor.

15. The method according to claim 12 wherein said composition comprises functional domains of asteroid hormone receptor selected from the group consisting of a glucocorticoid receptor, a thyroid receptor, a mineralocorticoid receptor, an estrogen receptor, an estrogen-related receptor, a retinoid receptor, an androgen receptor and a progesterone receptor.

16. The method according to claim 12 wherein the molar ratio, with respect to AP-1, of each of said ligand-binding domain or said DNA-binding domain in the composition falls in the range of about 0.5:1 up to 100:1.

17. A method to overcome the repression of expression of a gene product from a gene in an expression system having a steroid hormone present, when said gene is subject to negative regulation by steroid hormone receptors, said method comprising:

exposing said system to a compound or condition which induces Activator Protein-1 (AP-1) expression, wherein said AP-1 expression suppresses the repression of expression of said gene product.

18. The method according to claim 17 wherein said gene is a pro-opiomelanocortin gene, a prolactin gene, a proliferin gene, a chorionic gonadotropin alpha-subunit gene, a phosphoenolpyruvate carboxykinase gene, or a collagenase gene.

19. A method to overcome the inhibition of proliferation of cultured lymphoid

cells by asteroid hormone in the presence of asteroid hormone receptor, said method comprising:

exposing said lymphoid cells to compounds or conditions which induce Activator Protein-1 (AP-1) expression, wherein said AP-1 expression suppresses the inhibition of proliferation of said lymphoid cells.

20. A method for selecting a compound which disrupts the function of Activator Protein-1 (AP-1), said method comprising:

1) incubating a test compound in an assay system comprising a suitable growth medium and a cell line that expresses, in said suitable growth medium:

(i) steroid hormone receptor,

(ii) AP-1, and

(iii) AP-1-responsive reporter;

2) detecting AP-1 responsive reporter expression; and

3) selecting a compound which decreases expression of said AP-1 responsive reporter, thereby selecting a compound which disrupts the function of AP-1.

21. A method for selecting a compound which disrupts the function of Activator Protein-1 (AP-1), but does not affect the transcriptional activation of steroid hormone-responsive genes, said method comprising:

1) incubating a test compound which disrupts the function of AP-1 in an assay system comprising a suitable growth medium and a cell line that expresses, in said suitable growth medium:

(i) steroid hormone receptor, and

(ii) steroid hormone-responsive reporter; and

2) selecting a test compound which does not affect the transcriptional activation of steroid hormone-responsive genes, thereby selecting a compound which disrupts the function of Activator-Protein-1 (AP-1), but does not affect the transcriptional activation of steroid hormone-responsive genes.

22. A method to repress transcription activation of an Activator Protein-1 (AP-1)-responsive gene by AP-1 in an expression system that expresses an AP-1-responsive gone, said method comprising:

administering to said system:

(a) a composition comprising:

(i) functional ligand-binding domain of steroid hormone receptor, and

(ii) functional DNA-binding domain of steroid hormone receptor,

in an amount effective to repress expression of said AP-1-responsive gene, wherein said system comprises an endogenous compound that binds to said ligand binding domain.

Document Number 47

Entry 47 of 59 File: USPT Jun 17, 1997

DOCUMENT-IDENTIFIER: US 5639592 A
TITLE: Functional antagonism between proto-oncoprotein c-Jun and hormone receptors

ABPR:
Hormone receptors and the transcription factor Jun/AP-1 have been shown to reciprocally repress one another by a mechanism which is independent of DNA binding. For example, over-expression of AP-1 represses glucocorticoid-induced activation of genes carrying a functional glucocorticoid response element. Conversely, glucocorticoid has been shown to repress the transcriptional activation of genes which are controlled by promoters which contain the AP-1 binding site. In addition, methods are disclosed for selecting compounds useful for treating cells undergoing uncontrolled proliferation, such compounds being capable of disrupting the function of AP-1, but display substantially no ability to promote the transcriptional activation of hormone responsive genes.

BSPR:
This invention relates to steroid hormones, steroid hormone-like compounds, steroid hormone receptors, steroid hormone-like receptors, and related species. In a particular aspect, this invention relates to processes mediated by steroids and related hormones. In a further aspect, this invention relates to processes mediated by the proto-oncogenic protein complex, AP-1.

BSPR:
The AP-1 protein complex is a member of a class of nuclear proteins encoded by proto-oncogenes that have been implicated in diverse aspects of cell growth, differentiation, and development. The AP-1 binding site is recognized by c-Jun homodimers and c-Jun/c-Fos heterodimers. Binding of c-Fos to the AP-1 site is dependent on the formation of heterodimers with c-Jun. Homodimer and heterodimer formation is mediated through non-covalent interactions facilitated by a structure termed the leucine zipper. In addition to imparting positive regulatory effects on several pathways, the AP-1 complex has also been shown to confer negative regulation on several genes.

BSPR:
Up until now, the effect of a given protein on gene regulation has generally been thought to be the result of interaction between the protein and a regulatory element within the promoter region of the gene being regulated. Thus, compounds which exert an effect on more than one pathway are thought to recognize a responsive element which is common to more than one pathway. Consistent with this, Diamond et al., [in Science 249: 1266-1272 (1990)] describe studies employing a "composite" glucocorticoid response element (GRE), which binds selectively in vitro to both glucocorticoid receptor and c-Jun and c-Fos (components of the phorbol ester-activated AP-1 transcription factor). The authors then propose a general model for composite GRE action that requires DNA binding for interaction between receptor (i.e., glucocorticoid receptor) and non-receptor factors (i.e., c-Jun or c-Fos).

BSPR:
In accordance with the present invention, we have discovered that hormone receptors and transcription factor, AP-1, can reciprocally repress each other's transcriptional activation activity. Similarly, we have discovered that hormone receptors and transcription factor, AP-1, can reciprocally

derepress each other's ability to inhibit expression of certain genes. This is believed to occur via a novel mechanism which is independent of DNA binding.

BSPR:
The present invention, therefore, provides means to control the transcription activation of hormone-responsive gene products, and/or AP-1 responsive gene products. In addition, the present invention provides means to screen for compounds that inhibit cell growth, but which do not promote differentiation of said cells.

DRPR:
FIG. 4 summarizes the results of a deletion study to determine domains of the glucocorticoid receptor which repress AP-1 induced expression and induce glucocorticoid-mediated transcription.

DRPR:
FIG. 9 summarizes the results of a deletion study to determine domains of the retinoic acid receptor which repress AP-1 induced expression by the collagenase promoter.

DRPR:
FIG. 10 presents gel retardation assays performed to determine the ability of RAR to repress the binding of c-Jun to an AP-1 binding site.

DRPR:
FIG. 11 presents gel retardation assays performed to investigate the ability of RAR to repress the binding of AP-1 to an AP-1 binding site.

DEPR:
In accordance with another embodiment of the present invention, there is provided a method for identifying compound(s) which disrupt the AP-1 response pathway, but which exert substantially no effect on steroid hormone or steroid hormone-like responsive pathways, said method comprising:

DEPR:
It is desirable that the expression systems employed in this embodiment of the present invention be responsive to steroid hormone or steroid hormone-like compounds, while at the same time being substantially non-responsive to the presence of c-Jun, c-Fos, or AP-1.

DEPR:
As with the preceding embodiment of the present invention, it is desirable for the expression systems employed in this embodiment of the present invention to be responsive to steroid hormone or steroid hormone-like compounds, while being substantially non-responsive to the presence of c-Jun, c-Fos, or AP-1.

DEPR:
In accordance with still another embodiment of the present invention, there is provided a method to repress, in an expression system, transcription activation of AP-1-responsive gene(s) by AP-1, or analogs thereof, said method comprising:

DEPR:
It is desirable that the expression systems employed in this embodiment of the present invention be responsive to c-Jun, c-Fos, or AP-1, while being substantially non-responsive to the presence of steroid hormone or steroid hormone-like compounds.

DEPR:
In accordance with a still further embodiment of the present invention, there is provided a compound which forms a first complex with asteroid hormone or steroid hormone-like receptor; wherein said first complex, in the presence of AP-1, disrupts the function of AP-1; and wherein said first complex is substantially unable to promote transcriptional activation of steroid hormone or steroid hormone-like responsive genes.

DEPR:
Hormone-mediated transcription activation has been elucidated for many hormones; and for some hormones, this mode of activation can effect many different genes. It is sometimes desirable to modulate this transcription activation. In accordance with the present invention, this can be accomplished by either exposing the system to compound(s) and/or

condition(s) which induce AP-1 expression, or by administering to the system a peptide comprising the leucine zipper region of c-Jun, or analogs thereof, in an amount effective to repress expression of the hormone responsive gene product.

DEPR:
Compounds which are capable of inducing the expression of AP-1 include compounds which induce tumor formation (e.g., phorbol esters), growth factors (e.g., EGF, FGF, CSF), cytokines (e.g., IL-1, IL-2), neuropeptides (e.g., somatostatin), neurotransmitters (e.g., acetylcholine), protein kinase c (and compounds capable of inducing protein kinase c, e.g., EGF, insulin, platelet-derived growth factor, alpha-1 andronergic agents, IL-1, IL-2, and the like), and the like.

DEPR:
Conditions which are capable of inducing the expression of AP-1 include exposure of the system to ultraviolet irradiation, gamma irradiation, heat shock, stress, and the like.

DEPR:
Alternatively, instead of inducing the expression of endogenous (or exogenous) AP-1, the invention process can be accomplished by administering effective amounts of the c-Jun leucine zipper region to the system. Administration of a peptide comprising this component can be accomplished in a variety or ways, e.g., by direct introduction of purified or semi-purified peptide composition containing the desired component; by inducing expression of a gene construct encoding the leucine zipper region; and the like.

DEPR:
The molar ratio of protein comprising the c-Jun leucine zipper region, relative to the molar amount of steroid hormone receptor present in the expression system can vary widely. Broadly, ratios in the range of about 0.5 up to 100:1 are useful. Preferably, ratios of AP-1 component (or derivatives thereof) to steroid hormone receptor will fall in the range of about 1 up to 20:1; with molar ratios in the range of about 5 up to 15:1 being the presently most preferred ratio.

DEPR:
Hormones are also known to exert negative regulation on certain processes. It is sometimes desirable to modulate this negative regulation. In accordance with the present invention, this can be accomplished by either exposing the system to compound(s) and/or condition(s) which induce AP-1 expression, or by administering to said system a peptide comprising the leucine zipper region of c-Jun, or analogs thereof, in an amount effective to suppress the hormone-mediated repression of expression of gene products.

DEPR:
The molar ratio of composition comprising the ligand binding domain and DNA binding domain, relative to AP-1 present in the expression system, can vary widely. Broadly, ratios in the range of about 0.5 up to 100:1 are useful. Preferably, ratios of composition to AP-1 will fall in the range of about 1 up to 20:1; with molar ratios in the range of about 5 up to 15:1 being the presently most preferred.

DEPR:
The method of the invention can be employed in a variety of ways, e.g., for treating disease states which are stimulated by AP-1. Such disease states include tumor formation (e.g., formation of lymphomas), arthritis, asthma, allergies, rashes, and the like.

DEPR:
Hormone receptors contemplated for use in the practice of the present invention include the intracellular steroid receptors, such as, for example, glucocorticoid receptor(s), retinoic acid receptor(s), vitamin D.sub.3 receptor(s), thyroid receptor(s), mineralocorticoid receptor(s), estrogen receptor(s), estrogen-related receptor(s), retinoid receptor(s), androgen receptor(s), progesterone receptor(s), arylhydrocarbon receptor(s) and the like. Presently preferred receptors include glucocorticoid receptor(s), thyroid receptor(s), mineralocorticoid receptor(s), estrogen receptor(s), estrogen-related receptor(s), retinoid receptor(s), androgen receptor(s) and progesterone receptor(s). The presently most preferred receptor for use in the practice of the present invention is the glucocorticoid receptor, because this receptor has been particularly thoroughly characterized.

DEPR:
In accordance with one embodiment of the present invention, a compound useful for treating abnormal cells can be identified by screening for compounds which meet the two criteria of disrupting the function of AP-1, but which fail to promote transcriptional activation of steroid hormone-responsive genes.

DEPR:
A convenient means to assess the ability of test compound to disrupt the function of AP-1 is to employ the test compound in an assay system comprising a cell line capable of expressing steroid hormone receptor, AP-1, and AP-1-responsive reporter. Cells which express endogenous receptor, AP-1 and AP-1-responsive reporter, or cells having an exogenous source of one or more of the above can be employed. Preferred cells to employ for this purpose are cells which do not have a "hormone response element" associated with the AP-1 responsive reporter.

DEPR:
If the compound is effective in disrupting the function of the AP-1 pathway, the AP-1 responsive reporter (a gene product which can be readily measured by conventional methods) will not be expressed. Conversely, if the compound fails to disrupt the AP-1 responsive pathway, the AP-1 responsive reporter will be expressed and can readily be measured.

DEPR:
The collagenase-CAT constructs and plasmid (AP-1).sub.5 -TKCAT have been described by Angel et al., (See Mol. Cell. Biol., 1: 2256-2266 (1987).

DEPR:
c-Jun and GR expression plasmids were co-transfected into NIH3T3 cells and assayed to see whether c-Jun was able to inhibit GR-mediated activation of a GRE.sub.2 -TKCAT reporter plasmid [Schule et al., Science 242: 1418-1420 (1988)]. NIH3T3 cells were used in this experiment because they contain endogenous GR and upon starvation express only residual amounts of the AP-1 complex. As shown in FIG. 1, GR strongly induced reporter activity upon the addition of the synthetic glucocorticoid dexamethasone.

DEPR:
Co-transfection was carried out with constant amounts of GR plasmid expression and various amounts of c-Jun expression plasmid, .DELTA.Jun, a construct lacking the c-Jun coding sequences and .DELTA.RKJun, a construct lacking the c-Jun DNA binding domain, were also included in the Experiment. In FIG. 1, the cells are shown as either untreated (black bars) or treated with DEX (striped bars). Numbers presented in the Figure indicate g of co-transfected plasmid DNA. As shown in FIG. 1A, GR strongly induced reporter activity upon the addition of the synthetic glucocorticoid dexamethasone (DEX) (FIG. 1A, lane 1). Co-transfection of increasing amounts of c-Jun expression plasmids inhibited hormone-induced reporter activity in a concentration-dependent manner (FIG. 1A, compare lanes 1 with 2-5). In contrast, transfection of parental plasmid .DELTA.Jun, which lacks c-Jun coding sequences, did not alter the activity of GRE.sub.2 -TKCAT (FIG. 1A, lane 7). Because the GRE.sub.2 -TKCAT reporter lacks an intrinsic AP-1 site, inhibition does not appear to require binding of c-Jun to DNA. This was corroborated by a mutant, .DELTA.RKJun lacking a functional DNA binding domain, (FIG. 1A, lane 6). This mutant produced levels of repression similar to that of the wild-type c-Jun protein (FIG. 1A, compare lanes 5 and 6). The expression of control plasmid TKCAT, lacking the GRE, was not influenced by either hormone treatment or over-expression of c-Jun. c-Jun also repressed the hormone dependent activation of GRE.sub.2 -TKCAT by endogenous GR present in NIH3T3 cells (FIG. 1B, compare lanes 1 AND 2).

DEPR:
In this example, the ability of the GR to inhibit induction of an AP-1 responsive promoter was examined. The AP-1 inducible reporter construct (AP-1).sub.5 -TKCAT was transfected into GR negative CV-1 cells cultured in low (0.5%) serum (FIG. 2A). FIG. 2A summarizes the activity of reporter construct (AP-1).sub.5 -TKCAT and the control plasmid TKCAT in untreated (shown as black bars) or DEX treated (shown as striped bars) CV-1 cells co-transfected with either GR expression plasmid or parental vector .DELTA.GR lacking the GR coding sequences, and Jun/Fos expression plasmids. The CV-1 cells were cultured in low (0.5%) serum. This promoter has a high basal activity which is further stimulated (FIG. 2A, lane 2) by the cotransfection of Jun/Fos expression vectors. The presence of DEX and GR leads to potent inhibition of this induction (lane 2), whereas the control

plasmid .DELTA.GR, lacking the GR coding sequences, has no effect on Jun/Fos induction in the presence of DEX (lane 3). As shown with the TKCAT control (lanes 4 and 5), induction is dependent on the presence of the AP-1 sites and, in the absence of these sites, glucocorticoids alone have no effect.

DEPR:
The collagenase promoter provided an opportunity to examine this potential regulation of a cellular gene by GR. This gene was chosen because glucocorticoids have been shown to negatively regulate its expression [See Brinckerhoff et al., Biochemistry 25: 6378-6384 (1988)]. Whereas Jun/AP-1 and factors stimulating the Jun/AP-1 pathway are known to positively induce its activity, various collagenase promoter-CAT reporter plasmids were transfected into HeLa or CV-1 cells, respectively. HeLa and CV-1 cells were used because collagenase is expressed in both cell lines [Angel et al., Mol. Cell. Biol. 7: 2256-2266 (1987)]. In addition, HeLa cells express endogenous GR activity. FIG. 2B shows transcriptional activity of various collagenase promoter-CAT deletion mutants and heterologous reporters in HeLa cells cultured in 10% serum in the absence (black bar) or presence of DEX (striped bar). In the Figure, "TK" refers to the thymidine kinase-CAT construct; "(AP-1).sub.5 TK" refers to a construct comprising five copies of the collagenase AP-1 site in front of TK-CAT; and ".DELTA.MGRE.sub.p " refers to a construct comprising a consensus GRE cloned into DMCAT. As shown in FIG. 2B (lanes 1-3) addition of DEX to HeLa cells resulted in a 4-5 fold repression of 1200 Col-CAT and 73 Col-CAT reporter activity respectively, whereas the activity of the reporter plasmid 63 Col-CAT remained unchanged. These results indicate that repression is mediated by DNA sequences located between position -73 and -63 in the collagenase promoter. This region has been shown to contain a TPA-inducible enhancer which is recognized by AP-1 complex. The DEX responsive reporter .DELTA.MGRE.sub.p -CAT was activated in a hormone-dependent manner, indicating that the repression of the collagenase promoter or a heterologous reporter is dependent on the presence of the AP-1 site (FIG. 2B, lanes 6).

DEPR:
The data shown in FIGS. 2 and 3 demonstrate that both activation and repression of the collagenase promoter and the TK reporter is dependent on the presence of transcriptional activity of the AP-1 site. The AP-1 site is the major enhancer in the promoter of the collagenase gene and the only enhancer of (AP-.sub.1).sub.5 -TKCAT. Thus, glucocorticoids may function as general modulators of AP-1 responsive genes.

DEPR:
Deletion of the entire DNA binding domain resulted in a complete loss of repression (lane 10, mutant .DELTA.428-490). Further analysis revealed that deletion of either the first zinc finger (lane 11, mutant .DELTA.420-451) or the second zinc finger (lane 12, mutant .DELTA.450-487) of the GR completely eliminated repression. Although the DNA binding domain is necessary for repression, it is not sufficient, since mutants expressing only this region (lane 7, mutant .DELTA.9-385/550* and lane 9, mutant .DELTA.9-385/515*) are also inactive. Substitution of the GR DNA binding domain for that of the yeast transcription factor GAL4 resulted in a mutant which failed to repress (lane 13, mutant GgalG), even though it is able to activate GAL4 responsive promoters in a hormone-dependent fashion. The importance of the GR DNA binding domain is unexpected because GR does not bind to the collagenase AP-1 site (as shown in FIG. 6).

DEPR:
To test for a potential physical interaction between c-Jun and GR, gel retardation assays were performed. These assays were performed with .sup.32 P-labeled oligonucleotide containing a palindromic GRE and extracts prepared from COS cells transfected with constructs expressing either GR (FIG. 6A, lanes 1, 3, and 4) or beta-galactosidase (FIG. 6A, lane 2). Competition reactions were performed using a 50-fold excess of unlabeled oligonucleotide containing the GRE.sub.p (FIG. 6A, lane 3) or the collagenase AP-1 binding site (FIG. 6A, lane 4).

DEPR:
GR (obtained by over-expression in COS cells) formed a specific, retarded complex with an oligonucleotide containing a palindromic GRE (FIG. 6A, lane 1). The GR-GRE complex, while efficiently competed by a 50-fold excess of unlabeled GRE (lane 3), was unaffected by competition with a 50-fold excess of an oligonucleotide containing the AP-1 site found in the collagenase promoter (lane 4). This result indicates that the GR does not bind to the collagenase AP-1 binding site.

DEPR:
In this example, those DNA sequences in the collagenase promoter (i.e., an AP-1 responsive promoter) that mediate repression by RA were examined. Thus, various collagenase-CAT reporter plasmids together with RAR.alpha. expression vectors were co-transfected into HeLa cells cultured in 10% serum in the absence (FIG. 8, black bars) or presence of RA (FIG. 8, striped bars).

DEPR:
These results indicate that repression is mediated by DNA sequences located between position -73 and -63 in the collagenase promoter. Thus, RAR, similar to GR, can inhibit induction of an AP-1 responsive promoter.

DEPR:
To further test the ability of RAR to inhibit induction of an AP-1 responsive promoter, the AP-1 inducible reporter construct (AP-1).sub.5 -TKCAT was transfected into HeLa cells. The high basal activity of this promoter is also repressed in the presence of RA and RAR.alpha. (FIG. 8, bar 4), whereas expression of the control TK promoter is not influenced by RA (FIG. 8, bars 5). As one might expect, the RA-responsive reporter .beta.RE-TKCAT was activated in a hormone dependent manner (FIG. 8, bars 6).

DEPR:
The data shown in FIG. 8 demonstrate that repression of the collagenase promoter or heterologous reporter by RA depends on the presence of the AP-1 site.

DEPR:
The above experiments demonstrate that Jun/AP-1 activation can be efficiently repressed by RAR.alpha. in a hormone dependent manner. To define regions of the receptor involved in repression, several RAR mutants were analyzed in co-transfection studies in CV-1 cells for their ability to repress (AP-1).sub.5 -TKCAT reporter activity. In FIG. 9, the scale above each receptor indicates amino acid numbers. The wild type RAR.alpha. consists of the N terminus (amino acids 1-80), the DNA binding domain (amino acids 81-153), and the ligand binding domain (amino acids 154-462). The deleted amino acids are indicated at left. RA dependent repression of 1200Col-CAT reporter activity obtained by co-transfection with 0.1 .mu.g of RAR.alpha. expression plasmids was set at 100%.

DEPR:
To test for a potential physical interactions between RAR and AP-1, gel retardation assays were performed. Bacterially expressed RAR.alpha. was unable to form a retarded complex with a .sup.32 P-labeled oligonucleotide containing the collagenase AP-1 site (FIG. 10, lane 2), indicating that RAR does not inhibit collagenase expression by directly binding to this sequence. In contrast, in vitro translated c-Jun formed a specific, retarded complex (FIG. 10, lane 3). Addition of increased amounts of bacterially expressed RAR.alpha. severely reduced the amount of complex formed in a dose-dependent fashion (FIG. 10, lanes 4-7), whereas mock-transformed BL21 bacterial lysate did not affect binding of c-Jun to DNA (FIG. 10, lanes 8-11).

DEPR:
Bacterially expressed RAR.alpha. also inhibited AP-1 DNA binding when HeLa cell extract was used as AP-1 source (See FIG. 11, comparing lanes 1 with 2-4). Addition of mock-transformed BL21 bacterial lysate did not affect complex formation (FIG. 11, lanes 5-8). As a control, gel retardation assays were performed using NF-1 activity present in HeLa cell extracts and an oligonucleotide containing an NF-1 binding site. Addition of increasing amounts of bacterially expressed RAR.alpha. had no effect on NF-1 binding activity, demonstrating the specificity of the inhibitory effect or RAR.alpha. on AP-1 DNA binding.

DEPC:
The Collagenase AP-1 Site Is Required for DEX Repression

DEPC:
The AP-1 Site in the Collagenase Promoter Is Required for RA-Mediated Repression

DEPC:

Interferes with AP-1 Binding Activity

DEPV:
(a) the ability to disrupt the function of AP-1, when said compound is employed in a first assay system comprising a cell line capable of expressing:

DEPV:
(a) testing said compound in a first assay system to determine the effect of said compound on the AP-1 responsive pathway; wherein said first assay system comprises a cell line capable of expressing:

DEPV:
exposing said system to compound(s) and/or condition(s) which induce AP-1 expression, effective to repress expression of said steroid hormone-responsive or steroid hormone-like-responsive gene(s).

DEPV:
exposing said system to compound(s) and/or condition(s) which induce AP-1 expression, in an amount effective to suppress the repression of expression of said gene product(s).

DEPV:
exposing said system to compound(s) and/or condition(s) which induce AP-1 expression, effective to suppress the inhibition of proliferation and function of said lymphoid cells.

DEPW:
(ii) AP-1, and

DEPW:
(ii) AP-1, and

CLPR:
1. A method for selecting a compound, said method comprising selecting a compound which disrupts the function of Activator Protein-1 (AP-1), as determined by a first assay system, but does not promote transcriptional activation of a steroid hormone responsive gene, as determined by a second assay system, wherein:

CLPR:
2. The method according to claim 1 wherein said compound forms a first complex with asteroid hormone receptor; wherein said first complex, in the presence of AP-1, disrupts the function of AP-1; and wherein said first complex is substantially unable to promote transcriptional activation of steroid hormone responsive genes.

CLPR:
3. The method according to claim 1 wherein said receptor is a glucocorticoid receptor, a retinoic acid receptor, a vitamin D.sub.3 receptor, a thyroid receptor, a mineralocorticoid receptor, an estrogen receptor, an estrogen-related receptor, a retinoid receptor, an androgen receptor, or a progesterone receptor.

CLPR:
4. A method for identifying a compound which disrupts an Activator Protein-1 (AP-1) response pathway, but which exerts no substantial effect on asteroid hormone response pathway, said method comprising identifying a compound which has both an inhibitory effect on AP-1-responsive expression, as determined by a first assay system, and no substantial effect on steroid hormone-responsive expression, as determined by a second assay system, wherein:

CLPR:
5. The method according to claim 4 wherein said receptor is a glucocorticoid receptor, a retinoic acid receptor, a vitamin D.sub.3 receptor, a thyroid receptor, a mineralocorticoid receptor, an estrogen receptor, an estrogen-related receptor, a retinoid receptor, an androgen receptor or a progesterone receptor.

CLPR:
9. The method according to claim 6 wherein said compounds or conditions which induce expression of AP-1 are compounds which induce tumor formation, growth factors, cytokines, neuropeptides, neurotransmitters, protein kinase

c, or compounds which induce protein kinase c; or conditions of ultraviolet irradiation, gamma irradiation, stress or heat shock.

CLPR:
12. A method to repress transcription activation of an Activator Protein-1 (AP-1)-responsive gene by AP-1 in an expression system that expresses an AP-1-responsive gene, said method comprising:

CLPR:
14. The method according to claim 12 wherein said composition comprises functional domains of asteroid hormone receptor selected from the group consisting of a glucocorticoid receptor, a retinoic acid receptor, a vitamin D.sub.3 receptor, a thyroid receptor, a mineralocorticoid receptor, an estrogen receptor, an estrogen-related receptor, a retinoid receptor, an androgen receptor and a progesterone receptor.

CLPR:
15. The method according to claim 12 wherein said composition comprises functional domains of asteroid hormone receptor selected from the group consisting of a glucocorticoid receptor, a thyroid receptor, a mineralocorticoid receptor, an estrogen receptor, an estrogen-related receptor, a retinoid receptor, an androgen receptor and a progesterone receptor.

CLPR:
16. The method according to claim 12 wherein the molar ratio, with respect to AP-1, of each of said ligand-binding domain or said DNA-binding domain in the composition falls in the range of about 0.5:1 up to 100:1.

CLPR:
20. A method for selecting a compound which disrupts the function of Activator Protein-1 (AP-1), said method comprising:

CLPR:
21. A method for selecting a compound which disrupts the function of Activator Protein-1 (AP-1), but does not affect the transcriptional activation of steroid hormone-responsive genes, said method comprising:

CLPR:
22. A method to repress transcription activation of an Activator Protein-1 (AP-1)-responsive gene by AP-1 in an expression system that expresses an AP-1-responsive gone, said method comprising:

CLPV:
(b) said determination by said first assay system comprises identifying a test compound which decreases expression of said AP-1-responsive reporter when said test compound is incubated in said first assay system, thereby identifying a compound which disrupts the function of AP-1;

CLPV:
exposing said system to compounds or conditions which induce Activator Protein-1 (AP-1) expression, wherein said AP-1 expression represses expression of said steroid hormone-responsive gene.

CLPV:
exposing said system to a compound or condition which induces Activator Protein-1 (AP-1) expression, wherein said AP-1 expression suppresses the repression of expression of said gene product.

CLPV:
exposing said lymphoid cells to compounds or conditions which induce Activator Protein-1 (AP-1) expression, wherein said AP-1 expression suppresses the inhibition of proliferation of said lymphoid cells.

CLPV:
2) detecting AP-1 responsive reporter expression; and

CLPV:
3) selecting a compound which decreases expression of said AP-1 responsive reporter, thereby selecting a compound which disrupts the function of AP-1.

CLPV:
1) incubating a test compound which disrupts the function of AP-1 in an assay system comprising a suitable growth medium and a cell line that

expresses, in said suitable growth medium:

CLPV:
2) selecting a test compound which does not affect the transcriptional activation of steroid hormone-responsive genes, thereby selecting a compound which disrupts the function of Activator-Protein-1 (AP-1), but does not affect the transcriptional activation of steroid hormone-responsive genes.

CLPW:
(ii) AP-1, and

CLPW:
(ii) AP-1, and

CLPW:
(ii) AP-1, and

ORPL:
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Document Number 9

Entry 9 of 59 File: USPT Dec 21, 1999

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DOCUMENT-IDENTIFIER: US 6004748 A

TITLE: Method of inhibiting transcription utilizing nuclear receptors

DATE-ISSUED: December 21, 1999

INVENTOR-INFORMATION:

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APPL-NO: 8/ 757349
DATE FILED: November 27, 1996

PARENT-CASE:
This application is a continuation of U.S. Ser. No. 08/182,735, filed Jan. 14, 1994, now U.S. Pat. No. 5,643,720, which is a continuation of U.S. Ser. No. 08/032,726, filed Mar. 16, 1993, now abandoned, which is a continuation of U.S. Ser. No. 07/595,582, filed Oct. 10, 1990, now abandoned.

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FIELD-OF-SEARCH: 435/6, 530/358, 530/399, 536/23.1, 536/24.1

REF-CITED:

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ART-UNIT: 185

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ABSTRACT:

This invention provides a method of inhibiting the transcription of a gene, which is activated by AP-1 or an AP-1 component, comprising binding AP-1 or the component with a nuclear receptor so as to prevent the binding of AP-1 to the gene. The nuclear receptor can be the retinoic acid receptor, glucocorticoid receptor, vitamin D3 receptor, thyroid receptor, or estrogen receptor. Also provided is a composition of matter comprising AP-1 or an AP-1 component bound to a nuclear receptor. These methods and compositions can be used to treat arthritis and cancer.

22 Claims, 6 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 6

BRIEF SUMMARY:

BACKGROUND OF THE INVENTION

References are cited throughout the specification. These references in their entirety are incorporated by reference into the specification to more fully describe the state of the art to which it pertains.

Positive and negative control of gene expression by retinoids is mediated by nuclear receptors that are part of a large family of regulatory proteins including the steroid and thyroid hormone receptors, the vitamin D receptor, and the retinoid receptors (RAR). This latter group comprises three retinoic acid (RA) receptors: RAR.alpha., .beta. and .tau., that contain highly conserved DNA and ligand binding domains. In addition a more distantly related receptor, RXR, appears to be activated by a RA metabolite.

Nuclear receptors function as transcriptional activators in the presence of their ligand. RARs, like the thyroid hormone receptors, do not require ligand binding for nuclear localization and specific DNA interaction. Until the subject invention, interaction with specific DNA sequences was thought to be essential for all regulatory functions of nuclear receptors.

Since glucocorticoids and RA are known to repress members of the collagenase family they have promise as therapeutic agents in rheumatoid arthritis where proteinases, such as collagenase and stromelysin, play an important role in joint destruction. Collagenase is the only enzyme known to cleave collagen, a major structural component of bone and cartilage destroyed by the enzyme. Retinoids inhibit the production of collagenase by synovial cells while the tumor promoter 12-0-tetradecanoylphorbol-13-acetate (TPA) and the inflammatory mediators interleukin 1 (IL1) and tumor necrosis factor alpha (TNF.alpha.) stimulate collagenase secretion and transcription.

Thus, while the RA and glucocorticoid nuclear receptors are known to repress members of the collagenase family, the mechanism of this repression was not

known. A logical presumption was that the repression relates to the receptors known ability to bind DNA. However, the present invention provides the surprising discovery that these nuclear receptors actually inhibit transcription through a protein/protein interaction with AP-1, a protein complex composed of Jun homodimers and Jun/Fos heterodimers. Hence, a major discovery that regulatory function of nuclear receptors are mediated by a mechanism that does not involve direct binding to DNA is provided. This discovery provides a mechanism through which arthritis and cancer can be treated.

SUMMARY OF THE INVENTION

This invention provides a method of inhibiting the transcription of a gene, which is activated by AP-1 or an AP-1 component, comprising binding AP-1 or the component with a nuclear receptor so as to prevent the binding of AP-1 to the gene. The nuclear receptor can be the retinoic acid receptor, glucocorticoid receptor, vitamin D3 receptor, thyroid receptor, or the estrogen receptor.

Also provided is a composition of matter comprising AP-1 or an AP-1 component bound to a nuclear receptor. These methods and compositions can be used to identify and screen for new ligands of nuclear receptors useful for treatment of arthritis and cancer.

DRAWING DESCRIPTION:

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1 shows that RAR.beta. represses TPA induced collagenase promoter activity in a RA dependent fashion.

FIG. 2 shows inhibition of RAR activity by cJun and cFos.

FIG. 3 shows that RARB does not bind to the collagenase promoter.

FIG. 4 shows inhibition of cJun binding by RAR.beta. and RAR.tau. on synthetic AP-1 site.

FIG. 5 shows inhibition of RAR binding to RAR.epsilon. by cJun protein.

FIG. 6 shows that TR.alpha. represses TPA induced collagenase promoter activity in a T3 dependent fashion.

DETAILED DESCRIPTION:

DETAILED DESCRIPTION OF THE INVENTION

The invention provides a method of inhibiting the transcription of a gene, which is activated by AP-1 or an AP-1 component, comprising binding of AP-1 or a component of it with a nuclear receptor so as to prevent the binding of AP-1 to the gene.

It is known that Jun and Fos protooncoproteins make up AP-1. Thus, in one embodiment, the AP-1 component is a Jun protein or a Fos protein or portions thereof which either individually or in combination with other components activate transcription through AP-1 responsive nucleotide sequences. Thus, by "AP-1" is meant any compound having the structure of "AP-1" necessary for the binding of AP-1 to its responsive element.

In addition, "nuclear receptor" means a receptor, such as retinoic acid receptor, glucocorticoid receptor, vitamin D3 receptor, thyroid receptor and estrogen receptor, or portions of these receptors, which retain the function of binding AP-1 or transcriptionally activating fragments of AP-1.

The retinoic acid receptor (RAR) includes RAR.epsilon., RAR.alpha. RAR.beta., RAR.tau. and the related RXR proteins. The thyroid receptor includes erbA-T, TR.alpha.-2 and TR.alpha.-2 variant.

The gene in which transcription is inhibited can be any gene which is transcriptionally activated by AP-1 or an AP-1 component. In one embodiment, the gene encodes collagenase.

Since AP-1 is a transcriptional activator of the gene encoding collagenase, and collagenase is one of the enzymes known to break down collagen, a component of

bone, the control of AP-1 mediated transcription can be utilized to treat arthritis. Likewise, since AP-1 is comprised of the protooncogene encoded products Jun and Fos, the control of AP-1 mediated transcription can be utilized to treat those cancers caused by AP-1, a Jun or Fos component or another oncogene that regualtes AP-1 activity. Examples of control of AP-1 mediated cancer include the overexpression of AP-1, the expression of mutated forms of AP-1 and the increased AP-1 activity caused by expression of oncogenes such as H-ras.

Typically, the nuclear receptor binds to its ligand, e.g. retinoic acid receptor to retinoic acid, prior to binding to AP-1 or an AP-1 component. Thus, the invention provides methods in which ligand/receptor binding is required. However, circumstances can exist where the receptor directly binds to AP-1 or an AP-1 component.

Applicant's discovery also provides a novel composition of matter comprising AP-1 or an AP-1 component bound to a nuclear receptor. Examples of the nuclear receptor include retinoic acid, glucocorticoid, vitamin D3, thyroid and estrogen. Retinoic acid receptor includes RAR.epsilon., RAR.alpha. and RAR.tau. and thyroid receptor includes erbA-T, TR.alpha.-2 and TR.alpha.-2 variant.

The invention also provides a method of promoting the transcription of a gene which is activated by AP-1 or an AP-1 component comprising preventing the binding of AP-1, or an AP-1 component, with a nuclear receptor thereby allowing AP-1 to bind the gene. Such a method, given the teaching of the subject application, could be carried out by a person skilled in the art.

The invention still further provides a method of screening a sample for ligands which bind to a nuclear receptor to form a complex which binds or interferes with AP-1 or an AP-1 component. The method comprises contacting the ligand receptor complex with AP-1 and determining the binding of the complex to AP-1, the presence of binding or interference with AP-1 activity indicating the presence of a ligand. The binding of the complex to AP-1 can result in an increased anti-cancer or anti-arthritis effect compared to a ligand known to bind a nuclear receptor. Thus, one can screen for ligands with increased specificity or affinity of the receptor/ligand complex for AP-1 or AP-1 components. These ligands can be made by standard organic synthesis and screened using the methods of the invention.

This invention provides the added discovery that there is a family of nuclear receptors, in addition to their function as DNA binding transcriptional activators, which regulate transcription through protein interactions, especially with AP-1. Thus, the invention is much more than individual receptors binding AP-1. The invention involves the discovery that the family of nuclear receptors can act as transcriptional regulators through protein/protein interactions.

EXAMPLE I

The AP-1 Site of the Collagenase Promoter is Repressed by RARs

The region of the collagenase promoter that confers repression by RA is located between residues -73 and -63, which contain the AP-1 site that is responsible for induction by TPA, TNF.alpha. and IL1 (Angel et al., Mol. Cell. Biol. 7:2256-2266 (1987a); Brenner et al., Nature 337:661-663 (1989); Lafyatis et al., Mol. Endo. 4:973 (1990)). In addition, this site confers repression by GR as shown in Example VI.

The -73 Col CAT reporter gene was cotransfected into HeLa with an expression vector for RAR.beta./.epsilon.. Cells were grown in the presence of the indicated amounts of RA and/or TPA. Repression of TPA collagenase promoter activity is observed only in the presence of RAR and is dependant on the concentration of RA.

When the -73Col-CAT construct was transfected into Hela cells, grown in the presence and absence of RA, no substantial RA-dependent decrease in its basal or TPA induced CAT activity was observed. However, when an RAR.beta. expression vector was co-transfected with the -73Col-CAT construct, a dramatic repression of CAT activity was observed in the presence of RA, but not in the absence of RA. This repression was observed in either TPA-treated (FIG. 1) or untreated cells. Co-transfection of RAR.beta. with the -63Col-CAT showed no effect in the presence or absence of RA. The repression of -73Col-CAT by RAR.beta. is specific because the activity of another TPA-inducible promoter, that of cFos was not affected. Exchanging the RAR.beta. expression vector for the RAR.tau. vector yielded similar results. The repression of the TPA induced activity of -73 Col-CAT by RAR.beta. is concentration dependent: half maximal inhibition occurred at

10.sup.-9 M RA, while maximal repression required 10.sup.-6 M RA (FIG. 1). A similar concentration dependence was found for activation of an RAR.epsilon. reporter gene by RAR.beta.. Thus, the repression of collagenase transcription by RA is mediated by the AP-1 site and dependent on the level of RA activated RAR. This implies that the RARs either function by direct interaction with the AP-1 site and thereby interfere with AP-1 binding through a competitive mechanism, or alternatively by a mechanism that does not require direct binding of the RAR to the AP-1 site, but involves RAR-mediated interference with AP-1 activity. Despite a previous report that RA may repress collagenase expression by inhibition of cFos induction (Lafyatis et al., 1990 supra), we have not been able to find any effect of RA and RARs on the cFos promoter, in either HeLa (FIG. 1) or F9 cells.

To further demonstrate the involvement of AP-1 in repression of collagenase transcription by RA, we transfected the -73 Col-CAT reporter into F9 cells which have very low endogenous AP-1 activity. Co-transfection of -73 Col-CAT with a cJun expression vector activates its transcription, while a cFos expression vector does not lead to significant activation. As previously shown (Chiu et al., Cell 54:541-552 (1988)) co-transfection with a combination of cJun and cFos expression vector leads to further activation of -73 Col-CAT expression. In either case, expression of -73 Col-CAT was inhibited by RA, and further inhibition was observed in the presence of co-transfected RAR.beta. expression vector. Thus, elevating the level of RAR expression in F9 cells increases the repression of AP-1 activity by RA. Inhibition occurs regardless whether AP-1 activity is due to cJun homodimers or cJun/cFos heterodimers.

EXAMPLE II

Jun and Fos Repress RAR Activity

Next, we investigated whether the functional antagonism between RAR and AP-1 activity is limited to the AP-1 target site or whether it can be extended to targets which are positively regulated by RARs.

F9 cells were transfected with the RA responsive reporter T3RE.sub.2 -CAT (2 .mu.g) in the presence of 8 .mu.g of an empty expression vector (RSV) or the indicated amounts (in .mu.g) of cJun or cFos expression vectors. After transfection, the cells were incubated in the absence or presence of 10.sup.-7 M RA for 24 hours before harvesting and determination of CAT activity. The T3RE.sub.2 -CAT reporter was co-transfected into F9 cells with 1 .mu.g of either a mutant cJun (cJM). cJun, cFos or a combination of cJun and cFos expression vectors. RA treatment was done as indicated above.

Co-transfection of the reporter T3RE.sub.2 -CAT that contains two RAR responsive elements in front of the HSV-TK promoter (Glass et al., Cell 54:313-323 (1988)), with increasing concentrations of either a cJun or a cFos expression vector, resulted in strong dose-dependent inhibition of its activation by RA (FIG. 2). Similar inhibition has also been obtained when a CAT reporter gene containing the RARE derived from the RAR.beta. promoter was used. Co-transfection of T3RE.sub.2 -CAT with cFos expression vector resulted in a more effective inhibition of its induction than a co-transfection with a cJun expression vector. No further inhibition was obtained by co-transfection with a combination of the cJun and cFos expression vectors. Hence, elevated levels of both AP-1 constituents can repress the activation of an RARE by RAR.

EXAMPLE III

RAR Does Not Bind the AP-1 Site, But Inhibits Jun DNA Binding

Recently, it was reported that AP-1 may repress osteocalcin induction by RA and vitamin D by binding to a site embedded within an RA and vitamin D response element (Schule et al., 1990a). We therefore investigated the possibility that RAR can bind specifically to the AP-1 site or neighboring sequences of the collagenase promoter.

In vitro synthesized glucocorticoid receptor (GR) and retinoic acid receptor (RAR) were incubated with .sup.32 P-labelled DNA fragment derived from -73 COL-CAT by Hind III and Bam HI digestion. Protein-DNA complex was analyzed by gel retardation assay. Control represents the binding of unprogrammed reticulocyte lysate.

Gel retardation experiments were carried out with a 60 bp DNA fragment containing the collagenase AP-1 site. While specific binding of cJun to this site (FIG. 3) or a synthetic AP-1 site was readily detected, RAR.beta. did not bind to either site (FIG. 3). As reported in Example VI, GR also did not bind to either probe

(FIG. 3). Thus, the binding of RAR to site overlapping the AP-1 site cannot account for repression of collagenase induction, neither does the AP-1 site constitute a minimal RAR recognition element as previously suggested (Schule et al., supra).

As an explanation for its ability to repress AP-1 activity, we examined the effect of RAR on AP-1 binding activity.

In vitro synthesized cJun protein was incubated with one-fold (1) or five-fold (5) excess of in vitro synthesized RAR.beta. or RAR.tau. protein at 37.degree. C. for 15 minutes. The effect of RAR.beta. or RAR.tau. or cJun binding was analyzed by gel retardation assay. As control the inhibition effect of GR on cJun binding is shown in FIG. 4.

When cell-free translated RAR.beta. or RAR.tau. proteins were mixed with bacterially produced or in vitro translated cJun, a significant inhibition of cJun binding to the AP-1 site was seen (FIG. 4). The Inhibition of cJun binding to the AP-1 site was dependent on the amount of RARs used, and was also observed in response to pre-incubation with in vitro synthesized GR (FIG. 4). As shown for the GRs in Example VI, this in vitro inhibition of cJun binding was ligand independent. The inhibition of Jun binding activity is a specific effect of each receptor because pre-incubation of the cell-free translated RAR.beta. with anti-RAR antibodies prevented this inhibition, while pre-incubation with anti-GR or preimmune serum had no effect. Likewise, the inhibitory activity of cell-free translated GR is inhibited by anti-GR antibodies, but not by anti-RAR or preimmune serum. Because of its increased stability, the cJun/cFos heterodimer exhibits stronger AP-1 binding activity than the cJun homodimer. We therefore investigated whether the binding of the heterodimers could also be inhibited by the RAR. DNA binding was strongly enhanced by the addition of in vitro synthesized cFos, while cFos by itself did not bind the AP-1 site. Addition of RAR resulted in a comparable degree of repression for both cJun and cJun/cFos binding to the AP-1 site.

EXAMPLE IV

Jun Inhibits RAR DNA Binding

The inhibition of cJun or cJun/cFos DNA binding by RAR results from an interaction between the two classes of proteins in which the DNA binding activities of both proteins are impaired. Additionally, AP-1 could interfere with RAR function by competing for RARE binding. We therefore also investigated whether cJun could bind to the RARE and/or inhibit RAR DNA binding.

In vitro synthesized RAR protein was incubated with .sup.32 P-labelled DNA fragment containing one copy of RARE. The binding of RAR on RARE was analyzed by gel retardation assay. In competition experiments, 50-fold excess of unlabelled RARE (S) or comparable length of non-specific oligonucleotides (N) were included in the binding reaction. C represents the binding of unprogrammed reticulocyte lysate in FIG. 5. To test the effect of cJun protein on RAR binding, excess amount of bacterial expressed Jun protein was incubated with RAR at 37.degree. C. for 15 minutes before the addition of DNA. When anti-cJun (a cJun) was used, it was incubated with Jun protein at room temperature for 45 minutes before mixing with RAR protein.

Specific binding of RAR.beta. to the synthetic RARE derived from the RAR.beta. promoter region is shown in FIG. 5. When bacterially produced cJun protein was pre-incubated with RAR.beta., it inhibited its DNA binding activity in a concentration dependent manner. However, cJun did not increase the retention of the labelled RARE or lead to appearance of new protein-DNA complexes (FIG. 5). This inhibition was specific as it was prevented by pre-incubation of cJun with anti-Jun antibodies.

EXAMPLE V

Thyroid Hormone Receptor Represses Collagenase Promoter Activity

In addition, applicants have discovered that the thyroid hormone receptor, in the presence of Thyroid hormone, binds AP-1 through a protein/protein interaction and inhibits the transcription of AP-1 activated genes. Specifically, TR.alpha. represses TPA induced collagenase promoter activity in a T3 dependent fashion. The -73 Col CAT reporter gene was cotransfected into CV-1 cells with an expression vector for RAR.beta./.epsilon.. Cells were grown in the presence of the indicated amounts of T3 and/or TPA. Repression of TPA collagenase promoter activity is dependant on the concentration of T3. These results are shown in FIG.

6.

EXAMPLE VI

Glucocorticoid Receptor Inhibits AP-1 Activity

Applicants have also shown that the glucocorticoid receptor (GCR) is a potent inhibitor of AP-1 activity (Jun/Fos) and both c-Jun and c-Fos are potent repressors of GCR activity. In vitro experiments using purified GCR and c-Jun proteins showed that mutual repression is due to direct interaction between the two. Direct interaction between GCR and either c-Jun or c-Fos is demonstrated by cross-linking and coimmunoprecipitation. These findings also revealed a cross talk between two major signal transduction systems used to control gene transcription in response to extracellular stimuli, and a novel protein/protein interaction between the GCR and AP-1. The data demonstrating these findings is set forth in Yang-Yen et al., Cell 62:1205-1215, (1990).

Although the invention has been described with reference to the presently preferred embodiment, it should be understood that various modifications can be made without departing from the spirit of the invention. Accordingly, the invention is limited only by the following claims.

CLAIMS:

What is claimed is:

1. A method of inhibiting the transcription of a gene, which is activated by AP-1 or an AP-1 component, comprising binding AP-1 or the component with a nuclear receptor so as to prevent the binding of AP-1 to the gene, wherein transcription is inhibited.

2. The method of claim 1, wherein the AP-1 component is a Jun protein.

3. The method of claim 1, wherein the nuclear receptor is a glucocorticoid receptor.

4. The method of claim 1, wherein the nuclear receptor is a vitamin D3 receptor.

5. The method of claim 1, wherein the nuclear receptor is an estrogen receptor.

6. The method of claim 1, wherein the gene is the gene encoding collagenase.

7. The method of claim 1, wherein the receptor is bound to its ligand.

8. The method of claim 1, wherein the nuclear receptor is a retinoic acid receptor.

9. The method of claim 8, wherein the retinoic acid receptor is selected from the group consisting of RAR.epsilon., RAR.alpha. RAR.beta., RAR.tau., and RXR.

10. The method of claim 1, wherein the nuclear receptor is a thyroid receptor.

11. The method of claim 10, wherein the thyroid receptor is selected from the group consisting of erbA-T and TR.alpha.-2.

12. A method of inhibiting the transcription of a gene which is activated by a nuclear receptor which binds AP-1 or an AP-1 component comprising binding the receptor with AP-1 or an AP-1 component so as to prevent the binding of the nuclear receptor to the gene, wherein transcription is inhibited.

13. A composition of matter comprising AP-1 or an AP-1 component bound to a nuclear receptor.

14. The composition of claim 13, wherein the AP-1 component is selected from the group consisting of a Jun and a Fos protein.

15. The composition of claim 13, wherein the nuclear receptor is a glucocorticoid receptor.

16. The composition of claim 13, wherein the nuclear receptor is a vitamin D3 receptor.

17. The composition of claim 13, wherein the nuclear receptor is an estrogen

receptor.

18. The composition of claim 13, wherein the nuclear receptor is a retinoic acid receptor.

19. The composition of claim 18 wherein the retinoic acid receptor is selected from the group consisting of RAR.epsilon., RAR.alpha., RAR.beta. and RAR.tau. and RXR.

20. The composition of claim 13, wherein the nuclear receptor is a thyroid receptor.

21. The composition of claim 20, wherein the thyroid receptor is selected from the group consisting of erbA-T and TR.alpha.-2.

22. A method of promoting the transcription of a gene which is activated by AP-1 or an AP-1 component, comprising preventing the binding of AP-1 or an AP-1 component with a nuclear receptor thereby allowing AP-1 to bind the gene, wherein transcription is promoted.

Document Number 4

Entry 4 of 59 File: USPT Feb 15, 2000

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DOCUMENT-IDENTIFIER: US 6025388 A

TITLE: Method for inhibiting gene expression promoted by AP1 protein with RAR.beta. selective retinoids and method for treatment of diseases and conditions with such retinoids

DATE-ISSUED: February 15, 2000

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ART-UNIT: 164

PRIMARY-EXAMINER: Travers; Russell

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ABSTRACT:

Retinoid compounds which repress expression of the gene promoted by AP1 protein but which do not significantly activate expression of the genes having RA-responsive elements in their promoter region through RAR.alpha. and RAR.GAMMA. receptor subtypes, are used, with reduced side effects, for treating diseases and conditions which are responsive to therapy with retinoids.

18 Claims, 6 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 4

BRIEF SUMMARY:

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention is directed to a method of inhibiting gene expression promoted by the AP1 protein complex, with compounds which specifically or selectively transactivate RAR.beta. retinoid receptors. The present invention is also directed to a method of administering pharmaceutical compositions for the treatment or prevention of certain diseases and conditions which comprise a compound or ligand capable of inhibiting gene expression promoted by the AP1 protein complex through RARs, and which compound specifically or selectively induces gene expression only through the RAR.beta. retinoid receptors. The present invention is further directed to a method of selecting compounds of beneficial retinoid-like activity by assaying candidate compounds for ability to antagonize the gene expression medicated through the AP1 protein complex to surpress the expression of gene promoted by the AP1 protein complex, and for the ability, or lack thereof of the compounds to transactivate gene expression through RAR.alpha., RAR.beta. and RAR.GAMMA. receptors.

2. Related Art

Compounds having retinoic acid like (retinoid-like) biological activity have been known for a long time, and are described in numerous United States and foreign patents and scientific publications. Generally speaking, it has been established and accepted in the art that retinoic-acid like compounds (retinoids) are useful in humans and domestic animals for the treatment or prevention of many diseases

and conditions, as regulators of cell proliferation and differentiation, and particularly as agents for treating dermatoses, such as acne, Darier's disease, psoriasis, icthyosis, eczema and atopic dermatitis, and for treating and preventing malignant hyperproliferative diseases such as epithelial cancer, breast cancer, prostatic cancer, head and neck cancer and myeloid leukemias, for reversing and preventing atherosclerosis and restenosis resulting from neointimal hyperproliferation, for treating and preventing other non-malignant hyperproliferative diseases such as endometrial hyperplasia, benign prostatic hypertrophy, proliferative vitreal retinopathy and dysplasias, for treating autoimmune diseases and immunological disorders (e.g. lupus erythematosus) for treating chronic inflammatory diseases such as pulmonary fibrosis, for treating and preventing diseases associated with lipid metabolism and transport such as dyslipidemias, for promoting wound healing, for treating dry eye syndrome and for reversing and preventing the effects of sun damage to skin.

A classic measure of retinoic-acid like activity involves the effects of retinoic acid on ornithine decarboxylase. The original work on the correlation between retinoic acid and decrease in cell proliferation was done by Verma & Boutwell, Cancer Research, 1977, 37,2196-2201. That reference discloses that ornithine decarboxylase (ODC) activity increased precedent to polyamine biosynthesis. It has been established elsewhere that increases in polyamine synthesis can be correlated or associated with cellular proliferation. Thus, it was recognized early in the art that if ODC activity could be inhibited, cell hyperproliferation could be modulated. Although all causes for ODC activity increases are unknown, it is known that 12-O-tetradecanoylphorbol-13-acetate (TPA) induces ODC activity. Retinoic acid inhibits this induction of ODC activity by TPA. An assay essentially following the procedure set out in Cancer Res: 1662-1670,1975 has been extensively used in the art to demonstrate inhibition of TPA induction of ODC by tests compounds, which compound, if found inhibitory in the assay, is considered a "retinoid". Several other assays also exist in the art to determine if a compound has retinoid-like biological activity.

The use of retinoids in therapy of humans or domestic animals, is however, usually not without unpleasant or even serious side effects. Therefore, efforts have been made in the art to develop compounds which retain the beneficial activity of retinoids, but nevertheless lack the undesired side effects. U.S. Pat. No. 5,324,840 (assigned to the same assignee as the present application) for example discloses compounds which have retinoid-like activity but either lack, or have diminished skin-toxicity or teratogenic activity. An application for United States patent titled "Method of Treatment with Compounds Having Selective Agonist-like Activity on RXR Retinoid Receptors" Ser. No. 08/016,404 has been allowed and is expected to issue.

Significant efforts have also been made in the prior art to elucidate, on a biological, pharmacological or molecular level, the mechanisms by which retinoids act in living organisms, and specifically by which retinoids act to bring about beneficial therapeutic results and the undesired side effects. In connection with the foregoing it has been established in the prior art that one mode of action of retinoids is inducing gene expression through a class of receptors which are termed "RAR", another is through a class of receptors termed "RXR". Both the RAR and RXR receptor "families" have been shown to include several subtypes, which in the case of the RAR receptors are termed RAR.alpha., RAR.beta. and RAR.GAMMA.. Generally speaking, the following publications pertain to retinoid receptors and/or to compounds for selectively activating one or more of the receptor subtypes: D. J. Mangelsdorf et al. "Nuclear receptor that identifies a novel retinoic acid response pathway", Nature Vol 345 May 17, 1990 pp 224-229; and J. N. Rottman et al. A retinoic acid-responsive element in the apiloprotein AI gene distinguishes between two different retinoic acid response pathways, Molecular and Cellular Biology, July 1991, pp 3814-3820, M. Petkovich et al. "A human retinoic acid receptor which belongs to the family of nuclear receptor", Nature, Vol. 330, Dec. 3, 1987, pp 444-450; V. Giguere et al. "Identification of a receptor for the morphogen retinoic acid", Nature, Vol 330, Dec. 17, 1987, pp 624-629; N. Brand et al. "Identification of a second human retinoic acid receptor", Nature, Vol 332, Apr. 28, 1988, pp 850-853; A. Krast et al., "A third human retinoic acid receptor, hRAR", Proc. Natl. Acad. Sci. U.S.A., Vol 86, July 1989, pp 5310-5314; D. J. Mangelsdorf et al., "Characterization of three RXR genes that mediate the action of 9-cis-retinoic acid", Genes & Development, Vol. 6, 1992, pp. 329-344, D. J. Mangelsdorf et al. "Nuclear receptor that identifies a novel retinoic acid response pathway" Nature Vol. 345, May 17 1990, pp224-229, and International Publication WO 93 21146 (Ligand Pharmaceuticals) titled "Compounds Having Selectivity for Retinoid X Receptors".

As a still further development in the art pertaining to the mechanism of action of retinoids at the pharmacological and molecular level, it has been discovered

that in the presence of a proper ligand (retinoid compound), the RARs regulate gene expression either by directly binding to the RA-responsive element (RARE) or by antagonizing the action of c-Jun/c-Fos (AP1) protein complex. More specifically, in the action of a retinoid (ligand) that acts through the RARs, one or more of the three different RAR subtypes (RAR.alpha., RAR.beta. and RAR.GAMMA.) bind to the retinoid ligand. The resulting RAR-ligand complex regulates gene expression either by activating the expression of genes containing RAREs in their promoter regions, or by inhibiting the expression of certain genes by antagonizing AP1 protein complex (c-Jun/c-Fos, hereinafter "AP1 protein" or "AP1 protein complex") mediated gene expression. (See the articles: Mangelsdorf et al. (1994). The Retinoids; Biology, Chemistry, and Medicine, pp. 219-349. Raven Press Ltd., New York; Chambon, P. (1994) Semin, Cell Biol. 5, 115-125, and Pfahl. M. (1993) Endocr. Rev. 14, 651-658). Antagonizing the AP1 protein and thereby suppressing or inhibiting expression of the AP1-promoted gene is considered a second mechanism of action of retinoids. The latter is generally considered to be beneficial from a therapeutic standpoint, because the genes stimulated by the AP1 protein are involved in hyperproliferative and inflammatory diseases such as psoriasis, rheumatoid arthritis and tumor metastases. Published International Patent Application Nos. WO 92/05447 and WO92/07072 relate to the subject of inhibiting the gene expression which is stimulated by the AP1 protein complex.

In contrast to the action of retinoid compounds (ligands) to prevent or inhibit expression of the gene promoted by AP1, the transactivation of genes containing the RAREs, on the other hand, can lead to some of the undesired side-effect of retinoids. Therefore, a need exists in the prior art to separate the two types of retinoid actions, whereby retinoid drugs of lesser toxicity and therefore of greater therapeutic benefit may be obtained.

SUMMARY OF THE INVENTION

The present invention comprises a method for binding AP1 protein in a complex with a retinoid receptor RAR which has been activated by a retinoid compound or ligand, thereby inhibiting expression of the gene promoted by the AP1 protein, in preference over activating expression of genes which include the RAREs in their promoter regions. In other words, the present invention comprises a method for selectively inhibiting expression of the gene which is promoted by the AP1 protein, without practically inducing expression of genes that are normally triggered by retinoid like compounds through the RAREs.

In another aspect, the present invention comprises the above-mentioned gene regulation method employed for therapeutic purposes with reduced side effects, such as regulation of cell proliferation and differentiation, and particularly for treating dermatoses, such as acne, Darier's disease, psoriasis, icthyosis, eczema, atopic dermatitis, and for treating and preventing malignant hyperproliferative diseases such as epithelial cancer, breast cancer, prostatic cancer, head and neck cancer and myeloid leukemias, for reversing and preventing artherosclerosis and restenosis resulting from neointimal hyperproliferation, for treating and preventing other non-malignant hyperproliferative diseases such as endometrial hyperplasia, benign prostatic hypertrophy, proliferative vitreal retinopathy and dysplasias, for treating autoimmune diseases and immunological disorders (e.g. lupus erythematosus), for treating arthritis, asthma, allergies, chronic inflammatory diseases such as pulmonary fibrosis, for treating and preventing diseases associated with lipid metabolism and transport such as dyslipidemias, for promoting wound healing, for treating dry eye syndrome and in reversing and preventing the effects of sun damage to skin.

In still another aspect, the present invention comprises pharmaceutical compositions with which the above-noted methods are practiced, and which contain retinoid-like compounds capable of inhibiting AP1-promoted gene expression without causing expression of practical significance of genes having RAREs in their promoter region.

In yet another aspect, the present invention comprises a method for selecting retinoid-compounds as candidates for drugs having reduced side effects, by assaying a candidate test compound for its ability to repress AP1-protein promoted gene expression through the three RAR subtypes (RAR.alpha., RAR.beta. and RAR.GAMMA.) and for assaying the candidate test compound for its ability to transactivate gene expression through one or more of the three RARs. Compounds which inhibit AP1-protein expression through all three RAR subtypes, but transactivate only through the RAR.beta. receptor are selected, because the RAR.beta. receptor is practically not present in mammalian skin, and therefore side effects in the skin by the use of such compound are practically avoided.

In a further aspect, the present invention relates to the method of using the compounds which have the foregoing properties for cancer chemotherapy.

DRAWING DESCRIPTION:

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a graph showing data and the calculation of EC.sub.50, obtained in the Cationic Liposome Mediated Transfection Assay on the RAR.sub..alpha. receptor, with an example test compound and with the reference compound trans retinoic acid;

FIG. 2 is a graph showing the effect of varying concentrations of 4-[2-[[2-t-butyl-1-(2-tetrahydropyranoxy)]-4-phenyl]ethyn-1-yl]benzoic acid (Compound 2 AGN 192156) in the retinoid-mediated AP1 antagonism assay;

FIG. 3 is a graph showing the effect of varying concentrations of 4-[2-[[2-t-butyl-1-(2-tetrahydropyranoxy)]-5-phenyl]-ethyn-1-yl]-1-benzoic acid (Compound 7 AGN 192326) in the retinoid-mediated AP1 antagonism assay;

FIG. 4 is a graph showing the effect of varying concentrations of 6-[2-[[2-t-butyl-1-(2-tetrahydropyranoxy)]-5-phenyl]ethyn-1-yl]nicotinic acid (Compound 8 AGN 192327) in the retinoid-mediated AP1 antagonism assay;

FIG. 5 is a graph showing the effect of varying concentrations of 4-[[5,6,7,8-tetrahydro-5(RS)-(2'(RS)-tetrahydropyranyloxy)-8,8-dimethylnap hth-3-yl]ethynyl]benzoic acid (compound 13 AGN 192509) in the retinoid-mediated AP1 antagonism assay, and

FIG. 6 is a graph showing the effect of varying concentrations of 4-[[5,6,7,8-tetrahydro-5(RS)-(2'(SR)-tetrahydropyranyloxy)-8,8-dimethylnap hth-3-yl]ethynyl]benzoic acid (Compound 14 AGN 192508) in the retinoid-mediated AP1 antagonism assay.

DETAILED DESCRIPTION:

DETAILED DESCRIPTION OF THE INVENTION

It has been discovered in accordance with the present invention that the beneficial therapeutic and undesired toxic or side-effects of certain retinoid-like compounds can be pharmacologically separated in a practical sense, so that administration of pharmaceutical compositions containing such compounds to mammals, including humans, for therapeutic purposes results in beneficial therapeutic activity with significantly reduced side effects or toxicity.

More specifically, retinoid compounds are designed and discovered in accordance with the present invention which inhibit expression of gene promoted by the AP1 protein through binding as a ligand to all three RAR receptor subtypes (RAR.alpha., RAR.beta. and RAR.GAMMA.). The resulting retinoid ligand-RAR complex interacts with the AP1 protein in such a manner that the AP1 protein promoted gene expression is inhibited or suppressed. The same compounds, designed or discovered in accordance with the present invention, however are specific or selective in their ability to induce gene expression through the RAR.beta. receptor, in preferance over RAR.alpha. and RAR.GAMMA. receptors. Mammalian skin, including human skin, is known to contain only insignificant amounts of RAR.beta. receptors. Therefore, when the compounds designed or discovered in accordance with the present invention are administered to a mammal, including a human, for therapeutic purposes, the beneficial effects of AP1 protein-promoted gene inhibition are attained, and the undesired side effects through transactivation are reduced or minimized. Particularly noteworthy in this regard are the lack of side effects in the skin, because skin irritation and other skin toxicity has been considered one of the major disadvantages of conventional therapy with retinoids. The compounds designed or selected in accordance with the present invention have the desired therapeutic effect in the skin because the AP1 protein inhibition occurs through all three RAR receptor subtypes, but substantially lack skin toxicity, because they transactivate only through the RAR.beta. receptor which is practically not present in the skin.

The ability of a "retinoid" compound or ligand to meet the above-described criteria and to be useful in the methods in accordance with the present invention is assayed and demonstrated in the following manner. First, a transactivation assay employing chimeric RAR.alpha., RAR.beta. or RAR.GAMMA. binding regions

attached to an estrogen-response-element-chloramphenicol-acetyl transferase-construct (ERE-CAT) is used to measure the ability of the test compound or ligand to trigger expression of a synthetic gene containing RARE in its promoter region. This assay is hereinafter referred to as the chimeric receptor transactivation assay which is explained and described in detail as follows.

Chimeric Receptor Transactivation Assay

The assay is performed substantially as reported by Feigner P. L. and Holm M. (1989) Focus, 11, 2 and is based on the following principles, the description of which is followed by specific instructions how to perform the assay.

Retinoic acid receptors are a member of the steroid/thyroid receptor super family and they contain domains which are interchangeable within individual receptors. Thus, plasmids for chimeric retinoid receptors containing estrogen DNA binding domain and estrogen response element chloramphenicol acetyl-transferase enzyme are constructed and are grown in specific cultured bacteria. These plasmids respectively code for chimeric RAR.sub..alpha., RAR.sub..beta., RAR.sub..GAMMA., and if desired for testing RXR.sub..alpha., receptor proteins, and for the chloramphenicol acetyl A transferase (CAT) enzyme protein. The bacteria with these plasmids are obtainable in accordance with the procedure set forth in the article titled "Nuclear Retinoic Acid Receptors: Cloning, Analysis, and Function", M. Pfahl et al., Methods in Enzymology 189, p256-270 (1990) which is incorporated herein by reference. The detailed procedure how to isolate the DNA plasmids from the respective bacteria is also set forth below in detail, in the form of specific instructions under the title "Supercoiled Plasmid Isolation".

Thus, in accordance with the test procedure, DNA plasmid which codes for one of the chimeric RAR.sub..alpha., RAR.sub..beta., RAR.sub..GAMMA., and if desired for RXR.sub..alpha. receptor proteins is transfected into cultures of HeLa cells. It is for this purpose that HeLa cells are grown in a medium during the first day of the assay detailed below as the "Cationic Liposome Mediated Transfection Assay". In the transfection procedure, which is performed during the second day of the transfection assay, the DNA plasmid coding for the CAT enzyme is also added to each cell culture, in addition to the respective chimeric RAR.sub..alpha., or RAR.sub..beta. etc. coding plasmid. As is known and will be readily understood by those skilled in the art, especially in view of the above-cited M. Pfahl et al. article, chimeric retinoid receptors involved in this assay include a ligand binding domain which recognizes and binds specific agonist molecules, such as retinoic acid and analogs. These chimeric protein receptors (which were constructed in accordance with the teachings of the M. Pfahl et al. article) also contain a DNA binding domain, which is capable of binding to the "estrogen response element" (a DNA fragment) attached to the DNA plasmid coding for the CAT enzyme. The nature of the interaction is such, that only if an agonist (such as retinoic acid or analog) is bound to the ligand binding domain of the respective RAR.sub..alpha., RAR.sub..beta., etc. receptor, only then is the receptor bound through its DNA-binding domain to the estrogen response element of the estrogen-response-element-chloramphenicol-acetyl transferase-construct (ERE-CAT). In other words, through multiple interactions, CAT enzyme is produced by the HeLa cell in this assay only if an appropriate agonist ligand binds to the ligand binding site of the respective retinoid receptor.

The estrogen response-element-chloramphenicol acetyl-transferase construct (ERE-CAT) is itself obtained in accordance with the procedure described in the article Ryssel G. U. et al. Cell, Volume 46, pp 1053-1061 (1986), which is incorporated herein by reference. This procedure per se is well known in the art. The specific detailed procedure how to isolate and obtain the estrogen-response-element chloramphenicol-acetyl-transferase-construct (ERE-CAT) from bacteria is described in the procedure titled "Supercoiled Plasmid Isolation".

In addition to the foregoing, Lipofectin (LF) is also added to each cell culture. The purpose of the Lipofectin is to facilitate transport of plasmids through the cell membrane. The Lipofectin used in the procedure is available commercially.

As it will be well understood by those skilled in the art, as a result of transfection with the respective DNA plasmid coding for RAR.sub..alpha., or RAR.sub..beta. etc. chimeric receptors and as a result of transfection with the ERA-CAT (which codes for the CAT enzyme as described above), the aforementioned plasmids are incorporated into the HeLa cells cultured in the assay. The retinoid receptor plasmids undergo transcription (into m-RNA) and subsequent translation into the corresponding chimeric receptor protein. Therefore, the Hela cell cultures obtained in this manner produce the respective RAR.sub..alpha.,

RAR.sub..beta., RAR.sub..GAMMA., or RXR.sub..alpha. chimeric receptor protein. As a result of transfection with the ERA-CAT, the cell cultures of this assay also contain the genetic information for manufacturing the CAT enzyme. However, as is noted above, the latter genetic information is not transcribed, and the CAT enzyme is not produced by the respective cell cultures of this assay, unless an appropriate agonist compound binds to and activates the respective RAR.sub..alpha., RAR.sub..beta., RAR.sub..GAMMA., or RXR.sub..alpha. chimeric receptor protein in the cell and this activated agonist-receptor complex binds to the estrogen response element of the ERE-CAT construct.

The assay procedure is continued by adding, on the third day of the assay, an appropriate reference compound and the test compound (agonist or prospective agonist) to the respective HeLa cell culture, preferably in varying concentrations. As a result of this addition, if the test compound is an agonist, it binds to the respective RAR.sub..alpha., RAR.sub..beta., RAR.sub..GAMMA., or RXR.sub..alpha. chimeric receptor protein, and consequently the genetic information which codes for the CAT enzyme is transcribed in the cell, whereby CAT enzyme is made by the cell.

After lysis of the cell, which is performed on the fourth day of the below-detailed assay procedure, the activity of CAT enzyme in aliquot portions of the lysate is measured. This is done by incubating the lysate with chloramphenicol and tritium labeled acetyl coenzyme A. As a final measurement, the amount of tritium labelled acetyl chloramphenicol, which is formed in the enzymatic reaction involving the CAT enzyme, is measured in a scintillation counter.

The reference compound is retinoic acid (all trans) for the assays involving the RAR.sub..alpha., RAR.sub..beta., and RAR.sub..GAMMA. receptors. The data obtained in the assay are evaluated and expressed as follows. For each test compound and for each subspecies of the RAR receptors a graph (or the mathematical equivalent of a graph) is prepared where the "counts per minute" (cpm) obtained in the scintillation counter measurements are plotted (on the y axis) against the concentration of the test compound. A similar graph (or mathematical equivalent) is prepared for retinoic acid. EC.sub.50 of the test compound is defined as that concentration of the test compound which provides 1/2 (50%) of the maximum cpm number (maximum CAT enzyme activity) obtained in the same receptor in the same assay with the reference compound retinoic acid. This is illustrated in the graph of FIG. 1. Test results obtained in this assay in connection with compounds in accordance with the invention, are expressed in EC.sub.50 numbers.

SUPERCOILED PLASMID ISOLATION

Large Scale 1L Prep

DNA Isolation

1. Place cells on ice for 15 minutes. Harvest the bacterial cells (E. coli) by spinning down in 250 ml nalgene tubes at 7 k rpm, 10 minutes at 4.degree. C. using JA14 rotor, Beckman J2-21 M centrifuge. Discard the supernatant.

2. To each cell pellet add 1.0 ml Solution I, vortex to resuspend the pellet. Transfer the 1.0 ml of cells from one bottle to another. Transfer this to a 50 ml Oakridge tube. Use 4 ml of Solution I and wash the bottles again transferring from one bottle to the next. Transfer this also into the Oakridge tube. Using a pipet bring up the total volume to 16 ml with Solution I and mix the solution. Transfer 8 ml to a second Oakridge tube. Store at room temperature for 5 minutes.

Solution I

50 mM glucose, 25 mM Tris-Cl pH8, 10 mM EDTA pH8

3. Add to each tube 18 ml of freshly prepared Solution II. Mix contents gently by inverting the tube several times. Store on ice for 10 minutes. After this time the liquid should be clear with no aggregates. (If there are clumps, then the cells were not resuspended well enough previously.)

Solution II

1% sodium dodecylsulfate (SDS), 0.2N NaOH (4 ml 10% SDS, 0.8 ml 10N NaOH, 35.2 ml water)

4. Add 12 ml, (or as much as will fit) of ice-cold Solution III. Mix the contents

of tube by inverting it sharply several times. A white flocculent precipitate should appear. Store on ice for 10 minutes.

Solution III

Prepare as follows: to 60 ml 5M potassium acetate, add 11.5 ml of glacial acetic acid and 28.5 ml water.

5. Centrifuge at 4.degree. C. in a Beckman J2-21M centrifuge, JA20 rotor, 17 k rpm for 30 minutes.

6. Pipet approximately 12 ml of supernatant from the Oakridge tubes into 6 baked Corex tubes. Add 0.6 volumes of isopropanol (7.2 ml) mix by inversion and store at room temperature for 15 minutes to precipitate DNA.

7. Warm Beckman centrifuge by spinning JA20 rotor at 14 k rpm for 15 minutes at 20.degree. C.

8. Pellet DNA at 20.degree. C. in the J2-21M centrifuge, JA20 rotor at 10.5 k rpm for 30 minutes (use adapters for corex tubes).

9. Pour off supernatant, dry inside of tube with pasteur pipet on a vacuum flask.

10. Dry in vacuum dessicator for 10 minutes (longer drying time will make it hard to dissolve pellet).

Purification of Plasmid DNA by Centrifugation to Equilibrium in CsCl Density Gradients

11. Dissolve pellet by adding 1 ml TE (10 mM Tris-Cl pH 8, 1 mM EDTA pH8) to each corex tube. Place tubes in 37.degree. C. water bath to help pellets to dissolve faster (15-30 minutes).

12. Transfer liquid from like batch into one tube. Bring volume to 8.5 ml with TE.

13. Add 100 .mu.l RNase, DNase free (2 U/.mu.l, source Boehringer Mannheim Biochemical (BMB)).

14. Add 400 .mu.l of 10 mg/ml Ethidium Bromide.

15. Add 9.0 g CsCl and mix using a pasteur pipet.

16. Add solution to two 13.times.51 mm Beckman polyallomer quick-seal centrifuge tubes.

17. Spin at 50 k rpm for 12 hours in Beckman ultracentrifuge, VTi65.2 rotor, 20.degree. C.

18. After ultracentrifugation, two bands of DNA should be visible. The upper band consists of linear bacterial DNA and nicked circular plasmid DNA. The lower band consists of closed circular plasmid DNA. Only the lower CsCl-banded DNA is removed from the tube with a 3-ml syringe fitted to an 21-gauge needle (Needle is inserted into the side of the tube and 1.5-2 ml is removed).

19. Preparation for second CsCl centrifugation:

(9 ml--vol 1st CsCl band)--number g CsCl

(9 ml--vol 1st band--100 .mu.l 10 mg/ml Ethidium Bromide--50 .mu.l RNase)--ml TE pH 8.0

Combine 1st band, TE, CsCl, RNase and EtBr.

20. Add solution to 2 quick-seal tubes.

21. Spin at 50 k for 12 hours or 60 k rpm for 4 hours in ultracentrifuge, VTi65.2 rotor, 20.degree. C.

22. Remove twice CsCl-banded DNA (lower band only) to a 5 ml Falcon snap tube (as in step 18).

Extraction of Ethidium Bromide

23. Under fume hood add an equal volume isoamyl alcohol, vortex, spin at room temperature at 1500 rpm in Beckman TJ-6 centrifuge for 3 minutes.

24. Transfer bottom aqueous layer to fresh tube. Repeat 3-4 times or until aqueous layer is clear (no pink color).

25. Transfer clear aqueous layer to Spectra/Por 3 dialysis tubing, mwco 3500. (Tie a knot in the bottom of tubing before clamping dialysis tubing.) Add liquid using a pasteur pipet. Clamp top or dialysis tubing. Using a rubber band suspend tubing in 2.8 L TE (28 ml 1M Tris-Cl, pH8, 5.6 ml 0.500M EDTA, pH8). Always handle dialysis tubing carefully, with gloves.

26. Dialyze aqueous phase against several changes of 2.8 L TE pH8 (1.times. 2-4 hours, overnight and 1.times. 2-4 hours the next day).

27. In the tissue culture hood transfer the dialyzed DNA into sterile microcentrifuge tubes. Label tubes and store at -20.degree. C.

Cationic Liposome-Mediated Transfection

Reference: Felgner, P. L., and Holm, M. (1989) Focus 11, 2.

Use Sterile Technique Throughout

Grow up HeLa or CV-1 cells in T-125 culture flask. Cells are passed twice a week usually on Monday and Friday (0.5 ml cells into 15 ml medium)

DAY 1: Plating Cells

1. Trypsinize and collect cells from T-162 cm.sup.2 culture flask. Count cells using a hemocytometer. Usually, this amount of cells is enough for sixteen 12-well plates.

2. Based on the cell number, dilute cells in medium (D-MEM low glucose, 10% fetal bovine serum (FBS), 2 mM Glu) to a concentration of 60,000 cells per well.

Example Cell Calculation

want 40,000 cells/well and 200 wells

have (X) cells/ml

therefore, 40,000 cells/well.times.200 wells-total # ml cells

(X) cells/ml

needed

Using a Nalge 250 ml Filter Unit Receiver add total #ml cells to medium and bring final volume to 200 ml. Mix well by pipetting.

3. Add 1.0 ml of cells per well using a sterile 12.5 ml combitip (setting 4). Shake plates back and forth (do not swirl). Incubate at 37.degree. C. in a humidified 5% CO.sub.2 environment overnight. Cells are about 40% confluent prior to transfection.

Transfection: DAY 2 Preparation DNA/Lipofectin Complex

1. Using 50 ml polystyrene tubes prepare Lipofectin (LF) and DNA separately. Determine vol of LF and DNA needed for 2 .mu.g LF/well, 500 ng ERE-CAT DNA /well, 100 ng ER/RAR DNA per well. Determine total volume needed for experiment. (DNA concentration will vary between each plasmid prep and the following calculations will need to be adjusted accordingly.)

_____ DNA (prep date) .mu.l/well # wells vol DNA
Vol _____ Opti-Mem .alpha. .beta. .tau. CAT LF
lot # .mu.l/well # wells .mu.l LF Vol Opti-Mem

_____

Separately dilute LF and DNA in Opti-Mem media to a volume of 25 ul.times.#wells: Vol Opti-Mem 1=(25 ul.times.#wells)-total vol. DNA or LF.

2. Add the diluted LF to the diluted DNA and swirl tube gently. Let sit room

temperature for 10 min.

3. Aspirate off the medium from the wells and wash 2.times. using 0.5 ml Opti-Mem I (sterile 12.5 ml combitip, setting 2).

4. Add the DNA/LF complex to vol of Opti-Mem: (450 .mu.l.times.# wells). Invert tube to mix. Using a sterile 12.5 ml combitip (setting 2) add 500 .mu.l to each well. Shake plates back and forth to mix, do not swirl.

5. Incubate the cells for 6 hours at 37.degree. C. in a humidified 5% CO.sub.2 incubator.

6. After 6 hours add 0.5 ml medium to each well (D-MEM low glucose, 20% FBS charcoal treated, 2 mM Glu) Use 12.5 combitip setting 2 and place back in the incubator.

DAY 3: Drug Addition

1. 18 hours after the start of transfection add retinoids in triplicate (10 .mu.l) using a sterile 0.5 ml combitip (setting 1) and incubate for 20-24 hours at 37.degree. C. in a humidified 5% CO.sub.2 environment. ##EQU1##

Example

Retinoids are dissolved in acetone to a conc. of 5 mM and further diluted to 1 mM in EtOH. If retinoids do not go into solution place tube in hot water for 5 seconds followed by vigorous vortexing. Each experiment may have a different dilution scheme. For 2 concentrations per order of magnitude use a 3.16-fold dilution as follows: To labeled sterile 12.times.75 mm tubes (Falcon 2063) add 1080 ul of 100% EtOH. Using the 1 mM solution transfer 500 ul to the next tube (316 .mu.M). Vortex and repeat the transfer to the next tube down the line. Some retinoids are light sensitive, especially RA and 13-cis RA, and should be used with a red or very dim light. Log in the amount of compound used.

EXAMPLE

_____ Drug Dilution Vol add to well Final: -log [conc.] _____ 5 mM (initial) 1 mM 10 5.0 316 .mu.M 10 5.5 100 .mu.M 10 6.0 31.6 .mu.M 10 6.5 10 .mu.M 10 7.0 3.16 .mu.M 10 7.5 1 .mu.M 10 8.0 316 nM 10 8.5 100 nM 10 9.0 31.6 nM 10 9.5 10 nM 10 10.0 3.16 nM 10 10.5 1.0 nM 10 11.0 _____

Day 4 Mixed Phase Cat Assay

1. Wash cells in 12 mm wells once with 0.50 ml 1.times. PBS (no Ca/Mg).

2. Using a 5 ml combipipet (setting 1) add 100 .mu.l of a ice cold 1% Triton, 1 mM Tris-Cl pH7.8, 2 mM EDTA pH8, DNase I. Prepared as follows:

_____ LYSIS BUFFER (hypotonic buffer)
_____ 2.0 mg DNase I (Sigma) 4.925 ml water 50.0 .mu.l 100% Triton X-100 (BMB Lot # 5.0 .mu.l 1M Tris--Cl pH 7.8 20.0 .mu.l 0.5M EDTA pH 8 5.0 ml _____

3. Place on ice for 60 minutes. Agitate occasionally.

4. Transfer 50 .mu.l lysate from 3 wells at a time using titertrek multichannel pipet with tips attached to channels #1, #3, #6 to 96 U-bottom well (Costar). Place (unused lysate) plates at -20.degree. C.

5. Using a 1.25 ml combipipet (setting 1) add 50 .mu.l premix per well, gently shake plates and incubate 37.degree. C. for 2 hours.

_____ Vol. per Vol per Blank reaction X.sub.--(# assays) = total vol. _____ 47.0 27.0 .mu.l buffer I (250 mM Tris--Cl pH 7.8, 5 mM EDTA (Date: 1.5 1.5 .mu.l 1 mM HCl *** 20.0 .mu.l 5 mM Chloramphenicol (make fresh in buffer I) Lot # 0.75 0.75 .mu.l 4 mM Acetyl CoA in water (make fresh) Sigma Lot # 0.80 0.80 .mu.l 3H--Acetyl CoA (New England Nuclear) # NET-290L, 200 mCi/mmol)

_____

6. Using a titertrek multichannel pipet add 100 .mu.l of 7M Urea into each reaction well to quench the reaction. Do six at a time (Urea-Mallincrokt AR)

7. Using a titertrek multichannel pipet transfer 200 .mu.l reaction mixture into a 5 ml plastic scintillation vial(Research Products International #125514). Do three reactions at a time. (Urea-Mallincrokt AR)

8. Add 1 ml 0.8% PPO/Toluene (3.2 g PPO/4 L Toluene) Vortex vigorously for 5 seconds and allow the phases to separate for 15 minutes. Count cpm for 2.0 min-Beckman LS 3801.

Toluene-Mallinckrodt ScintillAR

PPO=2,5 Diphenyloxazole-RPI Lot #A3071

Alternatively, another assay, known as the holoreceptor transactivation assay can also be used for measuring the ability of the test compound or ligand to trigger expression of the genes containing RARs in their promoter regions. This assay is based on principles similar to the chimeric receptor transactivation assay described above, and is described below.

Holoreceptor Transactivation Assay

CV1 cells (5,000 cells/well) were transfected with an RAR reporter plasmid .tangle-solidup.MTV-TREp-LUC (50 ng) along with one of the RAR expression vectors (10 ng) in an automated 96-well format by the calcium phosphate procedure of Heyman et al. Cell 68, 397-406. (8). For RXR transactivation assays, an RXR-responsive reporter plasmid CRBP II-TK-LUC (50 ng) along with one of the RXR expression vectors (10 ng) was used substantially as described by Heyman et al. above, and Allegretto et al. J. Biol. Chem. 268, 26625-26633. (8, 9). RXR-reporter contained DRI elements from human CRBP II promoter (see Mangelsdorf et al. The Retinoids: Biology, Chemistry and Medicine, pp 319-349, Raven Press Ltd., New York and Heyman et al., cited above) (1, 8). A .beta.-galactosidase (50 ng) expression vector was used as an internal control in the transfections to normalize for variations in transfection efficiency. The cells were transfected in triplicate for 6 hours, followed by incubation with retinoids for 36 hours, and the extracts were assayed for luciferase and .beta.-galactosidase activities. The detailed experimental procedure for holoreceptor transactivations has been described in Heyman et al. above, and Allegretto et al. cited above. (8, 9). The results obtained in this assay in connection with exemplary compounds in accordance with the present invention are also expressed in EC.sub.50 numbers, in analogy to the results in the chimeric receptor transactivation assay. The Heyman et al. Cell 68, 397-406, Allegretto et al. J. Biol. Chem. 268, 26625-26633, and Mangelsdorf et al. The Retinoids: Biology, Chemistry and Medicine, pp 319-349, Raven Press Ltd., New York are expressly incorporated herein by reference.

Another assay used to determine the ability of a potential ligand (test compound) to meet the criteria of the present invention, is the retinoid-mediated AP1 antagonism assay which is described as follows.

Retinoid-Mediated AP1 Antagonism Assay

The anti-AP1 properties of retinoids are determined by measuring their ability to inhibit AP1-dependent gene expression in HeLa cells by transiently contransfecting them with a reporter gene and a receptor expression vector. Since the DNA binding domain of the RARs is involved in the inhibition of AP1 dependent gene expression (Schule. et al. Proc. Natl. Acad. Sci. U.S.A., 88:6092-6096, 1991), holoreceptors of RARs (.alpha., .beta., and .GAMMA.) are used in transfection assays to quantitate the relative potency of retinoids in antagonism of AP1-dependent gene expression.

Recombinant Plasmids

The expression vectors for RARs (.alpha., .beta., and .GAMMA.) have been described (Allegretto. et al., J. Biol. Chem. 268:26625-26633, 1993). AP1-reporter plasmid construct Str-AP1-CAT was prepared by cloning -84 to +1 base pairs of rat stromelysin-1 promoter (Matrisian et al., 6:1679-1686, 1986) in Hind III-Bam HI sites of pBLCAT3 (Luckow et al, Nucl. Acids Res. 15:5490, 1987). This sequence of stromelysin-1 promoter contains an AP1 motif as its sole enhancer element (Nicholson, et al., EMBO J. 9:4443-4454, 1990). The promoter sequence was prepared by annealing two synthetic oligonucleotides 5'AGAAGCTT ATG GAA GCA ATT ATG AGT CAG TTT GCG GGT GAC TCT GCA AAT ACT GCC ACT CTA TAA AAG TTG GGC TCA GAA AGG TGG ACC TCG A GGATCCAG3' (SEQ ID NO.1) and 5'-CT GGATCC TCG AGG TCC ACC TTT CTG AGC CCA ACT TTT ATA GAG TGG CAG TAT TTG CAG AGT CAC CCG CAA ACT GAC TCA TAA TTG CTT CCA T AAGCTT CT-3' (SEQ ID No. 2) containing Hind III and Bam HI restriction sites at their ends.

Transfection of Cells and CAT Assays

For retinoid-mediated AP1-antagonism assay, HeLa cells grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS, Life Technologies, Inc.) are transfected using the cationic liposome-mediated transfection procedure (Felgner, et al., Focus, 11:2, 1989). Cells are plated 18 hours before transfection at about 40% confluence (40,000-50,000 cells/well) in a 12-well tissue culture plate (Costar, Massachusettes). Cells are transfected with 1 .mu.g of reporter construct Str-AP1-CAT and 0.2 .mu.g of human RAR .alpha., .beta. or .GAMMA. expression vectors, along with 2 .mu.g of Lipofectamine (Life Technologies, Inc.) for each well in a total volume of 500 .mu.l. DNA/Lipofectamine complexes obtained by mixing 2 .mu.g Lipofectamine/well, 1 .mu.g Str-AP1-CAT/well, and 0.2 .mu.g RAR expression vector in a 50 ml polystyrene tube are incubated with HeLa cells. DNA is precipitated with Lipofectamine for 30 minutes at room temperature before transfer to cells. Five hours post-transfection, 500 .mu.l of DMEM containing 20% charcoal treated FBS (Gemini Bioproducts, Inc. California) is added. All the transfections are performed in triplicate. Test retinoids (at 10.sup.-10 to 10.sup.-7 M concentrations) are added 18 hours post-transfection and 6 hours later the cells are treated with 12-O-tetradecanoyl phorbyl-13-acetate (TPA) to induce AP1 activity. Retinoids are dissolved in acetone to a concentration of 5 mM and further diluted from this stock solution using ethanol. The next day after washing with phosphate buffered saline without calcium and magnesium (Life Technologies, Inc.), the cells are harvested and lysed for 60 min with occasional agitation using a hypotonic buffer (100 .mu.l/well) containing Dnase I, Triton X-100, Tris-HCl and EDTA. CAT activity is assayed in 50 .mu.l of the lysed cell extract using [.sup.3 H]acetyl CoA (DuPont NEN) in a 96-well U-bottom plate (Costar, Massachusettes). The CAT activity is quantified by counting the amount of .sup.3 H-acetylated forms of chloramphenicol using a liquid scintillation counter.

The results of this assay can be expressed in percentage of inhibition of TPA induced Str-AP1-CAT by RARs and varying concentrations of a test compound. The result of this assay for a number of examplary compounds in accordance with the invention are shown in graphs appended to this application as drawing figures.

The Schule, et al. Proc. Natl. Acad. Sci. U.S.A., 88:6092-6096, 1991, Matrisian et al., 6:1679-1686, 1986), Luckow et al, Nucl. Acids Res. 15:5490, 1987), Nicholson, et al., EMBO J. 9:4443-4454, 1990), and Felgner, et al., Focus, 11:2, 1989) articles are hereby expressly incorporated by reference.

Another assay in which the ability of compounds in accordance with the present invention to inhibit AP1 protein promoted gene expression is tested and determined is described below. It has been observed that compounds designed or discovered in accordance with the present invention bind to all three RAR receptor subtypes (.alpha., .beta. and .GAMMA.), and remove the AP1 protein from its ability to promote gene expression, even though the same compound transactivates specifically or selectively only through the RAR.beta. subtype.

In Vitro RAR Binding Assay

For in vitro RAR binding experiments, baculovirus/Sf21 insect cell system was used to express human RAR.alpha., -.beta., and -.GAMMA. as described Allegretto et al. J. Biol. Chem. 268, 26625-26633 (9). Suspension-grown Sf21 cells were infected with the recombinant viruses at a multiplicity of infection of 2 for 48 hours, followed by disruption of the infected cells in 10 mM Tris, pH 7.6, 5 mM dithiothreitol, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 0.4 M KCl as described Heyman et al. Cell 68, 397-406, and Alegretto et al. cited above. (8, 9). The binding assay contained 5-20 .mu.g of extract protein along with [.sup.3 H]all-trans-retinoic acid (5 nM) and varying concentrations (0-10.sup.-5 M) of competing ligand in a 250 .mu.l reaction. The binding assays were performed substantially as described previously in Heyman et al. cited above, and Alegretto et al. cited above (8, 9). The results of this assay are expressed, as customary in the art, in KD.sub.50 numbers.

It follows from the foregoing description that compounds which can be used in the methods of selective suppression of AP1-promoted gene expression, and therefore as therapeutic agents of reduced skin toxicity, show significant binding in the in vitro RAR binding assay substantially to all three RAR subtypes, and inhibit AP1 protein promoted gene expression in the retinoid-mediated AP1 antagonism assay. These compounds however, show substantial transactivation in the chimeric receptor transactivation assay or in the holoreceptor transactivation assay only through the RAR.beta. receptor subtype. In other words, in the transactivation assays, the compounds do not show significant transactivation through RAR.beta.

and RAR.GAMMA. receptors. In as much as the compounds do show some transactivation through RAR.beta. receptors (which are not present in substantial amount in the skin), it is generally speaking a criterion for the compounds used in the invention that they should be at least approximately 20 times less active through the RAR.alpha. and RAR.GAMMA. receptors than through the RAR.beta. receptors.

Accordingly, the foregoing assays provide a method for selecting retinoid compounds as potential useful drugs having reduced skin toxicity. The drugs so selected or designed to meet the criteria of the invention, are then utilized for treatment in combination with conventional pharmaceutical excipients.

In determining appropriate formulations one skilled in the art will consider that the compounds utilized in accordance with this invention may be administered systemically or topically, depending on such considerations as the condition to be treated, need for site-specific treatment, quantity of drug to be administered, and numerous other considerations.

In the treatment of dermatoses, it will generally be preferred to administer the drug topically, though in certain cases such as treatment of severe cystic acne or psoriasis, oral administration may also be used. Any common topical formulation such as a solution, suspension, gel, ointment, or salve and the like may be used. Preparation of such topical formulations are well described in the art of pharmaceutical formulations as exemplified, for example, Remington's Pharmaceutical Science, Edition 17, Mack Publishing Company, Easton, Pa. For topical application, these compounds could also be administered as a powder or spray, particularly in aerosol form. If the drug is to be administered systemically, it may be confected as a powder, pill, tablet or the like or as a syrup or elixir suitable for oral administration. For intravenous or intraperitoneal administration, the compound will be prepared as a solution or suspension capable of being administered by injection. In certain cases, it may be useful to formulate these by injection. In certain cases, it may be useful to formulate these compounds in suppository form or as extended release formulation for deposit under the skin or intramuscular injection.

Other medicaments can be added to such topical formulation for such secondary purposes as treating skin dryness; providing protection against light; other medications for treating dermatoses; medicaments for preventing infection, reducing irritation, inflammation and the like.

Treatment of dermatoses or any other indications known or discovered to be susceptible to treatment by retinoid-like compounds will be effected by administration of the therapeutically effective dose of one or more compounds of the instant invention. A therapeutic concentration will be that concentration which effects reduction of the particular condition, or retards it expansion. In certain instances, the compound potentially may be used in prophylactic manner to prevent onset of a particular condition. A useful therapeutic or prophylactic concentration will vary from condition to condition and in certain instances may vary with the severity of the condition being treated and the patient's susceptibility to treatment. Accordingly, no single concentration will be uniformly useful, but will require modification depending on the particularities of the disease being treated. Such concentrations can be arrived at through routine experimentation. However, it is anticipated that in the treatment of, for example, acne, or similar dermatoses, that a formulation containing between 0.01 and 1.0 milligrams per milliliter of formulation will constitute a therapeutically effective concentration for total application. If administered systemically, an amount between 0.01 and 5 mg per kg per day of body weight would be expected to effect a therapeutic result in the treatment of many diseases for which these compounds are useful.

ASSAY RESULTS

Tables 2 and 3 and the graphs of FIGS. 2-6 show the results of some of the above described assays with exemplary compounds, in accordance with the invention. For the purposes of biological testing the compounds are assigned arbitrary designation numbers (AGN numbers) which are shown in the drawing figures. To facilitate comparison with the numbering of compounds in this application, Table 1 shows the correspondence between the "AGN number" and the "compound number" in this application.

TABLE 1 _____ Compound # AGN #

_____ 2 192156 7 192326 8 192327 13 192509 14 192508 _____

TABLE 2 EC.sub.50 (nanomolar) Compound # RAR.alpha. RAR.beta. RAR.GAMMA. Chimeric Receptor Transactivation Assay 2 0.0 47.0 0.0 7 2520 2.63 303 8 0.0 8.24 1590 Holoreceptor Transactivation Assay 13 0.0 69.0 0.0 14 0.0 90 0.0

0.0 in Table 2 indicates a value greater than 1000 nM

TABLE 3 In Vitro RAR Binding Assay KD.sub.50 (nanomolar) Compound # RAR.alpha. RAR.beta. RAR.GAMMA. 2 1178 295 509 7 95 108 103 8 505 468 391 13 129 17 63 14 171 75 104

As can be seen in Table 2 the compounds used in the methods of the invention are substantially inactive to transactivate, (trigger expression of genes which have RAREs in their promoter region) through RAR.alpha. and RAR.GAMMA. receptors. However, as Table 3 indicates, these compounds are still capable of binding with approximately the same strength to all three of the RAR.alpha., RAR.beta. and RAR.GAMMA. receptors. The latter is an indication of their ability to repress AP1 protein promoted gene expression. FIGS. 2-6 of the appended drawing figures show the results obtained with varying concentrations of the exemplary compounds 2, 7, 8, 13, and 14 in the retinoid mediated AP1 antagonism assay. These data also show, that even though the compounds do not transactivate significantly through the RAR.alpha. and RAR.GAMMA. receptors, they inhibit AP1 promoted gene expression through all three RAR receptor subtypes.

DESCRIPTION AND PREPARATION OF COMPOUNDS IN ACCORDANCE WITH THE PRESENT INVENTION

Compounds which, in accordance with the present invention are suitable for use in the method of AP1-promoted gene suppression and the resulting therapeutic applications, have the structure shown by Formula 1 and Formula 2, where X is N or CH, R is H or lower alkyl of 1 to 6 carbons; in Formula 1 the tetrahydropyranyloxy and the t-butyl groups are attached either to the 3 or the 4 position of the phenyl ring, and where the wavy lines represent a bond which is of either stereo-isomeric configuration that is possible for that bond. ##STR1##

In the description of the compounds the term alkyl refers to and covers any and all groups which are known as normal alkyl, branched-chain alkyl and cycloalkyl. Lower alkyl means the above-defined broad definition of alkyl groups having 1 to 6 carbons, and as applicable, 3 to 6 carbons for branch chained and cycloalkyl groups. The term "ester" as used here refers to and covers any compound falling within the definition of that term as classically used in organic chemistry. A pharmaceutically acceptable salt may be prepared for any compounds in this invention having a functionality capable of forming such-salt, for example an acid functionality. A pharmaceutically acceptable salt is any salt which retains the activity of the parent compound and does not impart any deleterious or untoward effect on the subject to which it is administered and in the context in which it is administered.

Pharmaceutically acceptable salts may be derived from organic or inorganic bases. The salt may be a mono or polyvalent ion. Of particular interest are the inorganic ions, sodium, potassium, calcium, and magnesium. Organic salts may be made with amines, particularly ammonium salts such as mono-, di- and trialkyl amines or ethanol amines. Salts may also be formed with caffeine, tromethamine and similar molecules. Where there is a nitrogen sufficiently basic as to be capable of forming acid addition salts, such may be formed with any inorganic or organic acids or alkylating agent such as methyl iodide. Preferred salts are those formed with inorganic acids such as hydrochloric acid, sulfuric acid or phosphoric acid. Any of a number of simple organic acids such as mono-, di- or tri-acid may also be used.

Some of the compounds utilized in accordance with the present invention may contain one (and may contain more than one) chiral center and therefore exist in enantiomeric and diastereomeric forms. Unless otherwise stated, the scope of the present invention is intended to cover all such isomers per se, as well as mixtures of diastereomers and racemic mixtures of enantiomers (optical isomers) as well.

Referring again to the compounds of Formula 1 and Formula 2, the R group is even more preferably H, or lower alkyl of 1 to 3 carbons.

Specific examples of compounds used in the methods of the present invention are disclosed in Table 4 with specific reference to Formula 1 and Formula 2.

TABLE 4 Position of THP.sup.1 position of Compound # Formula # on phenyl ring.sup.2 t-butyl group R X

2 1 4 3 H CH 7 1 3 4 H CH 8 1 3 4 H N 13 2 -- -- H.sup.3 -- 14 2 -- -- H.sup.4 --

.sup.1 THP stands for tetrahydropyranyloxy .sup.2 the ethynyl substituent occupies position "1" on the phenyl ring for the purposes of numbering in this table .sup.3 Assymetric centers of the (5RS) and 2(RS) configuration .sup.4 Assymetric centers of the 5(RS) and 2(SR) configuration

The compounds of Formula 1 and Formula 2 can be made by the synthetic chemical pathways illustrated here. The synthetic chemist will readily appreciate that the conditions set out here are specific embodiments which can be generalized to any and all of the compounds represented by these formulas. ##STR2##

Referring now specifically to Reaction Scheme 1, the t-butyl substituent group (indicated in the scheme as R.sub.1) is introduced by a Friedel-Crafts (or the like) reaction into the bromophenol compound of Formula 3, to yield the substituted bromophenols of Formula 4. The bromophenols of Formula 3 are commercially available thereby rendering the compounds of Formula 4 readily accessible to one of ordinary skill in the art as commercially available chemicals and/or through use of such Fridel Crafts alkylation (and the like) reactions which are well known in the art.

The compounds of Formula 4 are then reacted with 3,4-dihydro-2H-pyran (DHP) to provide the 2-tetrahydropyranoxy bromobenzenes of Formula 5. The latter reaction is typically conducted in an inert aprotic solvent, such as dichlomethane, under mildly acidic conditions, such as in the presence of pyridinium p-toluenesulfonate. The 2-tetrahydropyranoxy bromobenzenes of Formula 5 are thereafter reacted with trimethylsilylacetylene to provide the 2-tetrahydropyranoxy trimethylsilylethynylbenzenes of Formula 6. The reaction with trimethylsilylacetylene is typically conducted at moderate heat (approximately 55.degree. C.) in the presence of cuprous iodide, a suitable catalyst, typically having the formula Pd(PPh.sub.3).sub.2 Cl.sub.2, an acid acceptor (such as triethylamine) under an inert gas (argon) atmosphere. The 2-tetrahydropyranoxy trimethylsilylethynylbenzenes of Formula 6 are then reacted with base (potassium hydroxide or potassium carbonate) in an alcoholic solvent, such as methanol, to provide the tetrahydropyranoxy ethynylbenzenes of Formula 7. The ethynyl compounds of Formula 7 are preferably coupled directly with the aromatic or heteroaromatic reagent X.sub.1 --Y--B' (Formula 8) in the presence of cuprous iodide, a suitable catalyst, typically Pd(PPh.sub.3).sub.2 Cl.sub.2, an acid acceptor, such as diethylamine, under inert gas (argon) atmosphere. The symbol X.sub.1 in Formula 8 represents a halogen, preferably chloro or iodo; the symbol Y represents the phenyl or pyridyl ring shown in Formula 1; and B' represents the carboxylic acid or carboxylic acid ester shown in Formula 1, or a synthetic precursor of the same from which the carboxylic acid or carboxylic acid ester group can be readily obtained by synthetic steps well known by the practicing organic chemist. Alternatively, a zinc salt (or other suitable metal salt) of the compounds of Formula 7 can be coupled with the reagents of Formula 8 in the presence of Pd(PPh.sub.3).sub.4 or similar complex. Generally speaking, coupling between an ethynylbenzene compound or its zinc salt and a halogen substituted aryl or heteroaryl compound, such as the reagent of Formula 8, are described in U.S. Pat. No. 5,264,456, the specification of which is expressly incorporated herein by reference. The compounds of Formula 9 are the compounds of the invention defined by Formula 1, or such derivatives thereof protected in the B' group, from which the protecting group can be readily removed by reactions well known in the art. One such reaction employed for the synthesis of several exemplary compounds of this invention is saponification of an ester group to provide the free carboxylic acid or its salt.

The halogen substituted phenyl or pyridyl compounds of Formula 8 can, generally speaking, be obtained by reactions well known in the art. An example of such compound is ethyl 4-iodobenzoate which is obtainable, for example, by esterification of 4-iodobenzoic acid. Another example is ethyl 6-iodonicotinate which can be obtained by conducting a halogen exchange reaction on 6-chloronicotinic acid, followed by esterification. ##STR3##

Referring now to Reaction Scheme 2 a synthetic route leading to the compounds of Formula 2 is illustrated. In accordance with this scheme, a 7-bromo-3,4-dihydro-naphthalen-1(2H)-one of Formula 10 is the starting material. This compound is obtained from ethyl (4-bromophenyl)acetate in a series of reaction steps which are described in detail in the specific embodiments (experimental) section of this application. Thus, in accordance with Reaction Scheme 2 the compound of Formula 10 is reacted with trimethylsilylacetylene to

provide 7-trimethylsilylethynyl-3,4-dihydro-naphthalen-1(2H)-one (Formula 11). The reaction with trimethylsilylacetylene is typically conducted under heat (approximately 100.degree. C.) in the presence of cuprous iodide, a suitable catalyst, typically having the formula Pd(PPh.sub.3).sub.2 Cl.sub.2, an acid acceptor (such as diethylamine) under an inert gas (argon) atmosphere. Typical reaction time is approximately 24 hours. The 7-(trimethylsilyl)ethynyl-3,4-dihydro-naphthalen-1(2H)-one of Formula 11 is then reacted with base (potassium hydroxide or potassium carbonate) in an alcoholic solvent, such as methanol, to provide 7-ethynyl-3,4-dihydro-1-naphthalen-1(2H)one of Formula 12. The compound of Formula 12 is then coupled with the aromatic (phenyl) or heteroaromatic (pyridyl) reagent X.sub.1 --Y--B'(Formula 8, defined as in connection with Scheme 1) in the presence of cuprous iodide, a suitable catalyst, typically Pd(PPh.sub.3).sub.2 Cl.sub.2, an acid acceptor, such as triethylamine, under inert gas (argon) atmosphere. Alternatively, a zinc salt (or other suitable metal salt) of the compound of Formula 12 can be coupled with the reagents of Formula 8 in the presence of Pd(PPh.sub.3).sub.4 or similar complex. Typically, the coupling reaction with the reagent X.sub.1 --Y--B' (Formula 8) is conducted at room or moderately elevated temperature. The 7,8-dihydro-naphthalen-5(6H)-one derivatives of Formula 13 are reduced with a mild reducing agent such as sodium borohydride, to yield the corresponding 5-hydroxy-5,6,7,8-tetrahydronaphthalene derivatives of Formula 14. The compounds of Formula 14 are thereafter reacted with 3,4-dihydro-2H-pyran (DHP) to provide the 2-tetrahydropyranoxy derivatives of Formula 15. The latter reaction is conducted under conditions similar to the conditions described above in connection with Reaction Scheme 1, namely under mildly acidic conditions, such as in the presence of pyridinium p-toluenesulfonate. The diastereomeric tetrahydropyranoxy compounds can be separated by conventional techniques such as chromatography. The compounds of Formula 15 are the compounds of the invention defined by Formula 2, or such derivatives thereof protected in the B' group, from which the protecting group can be readily removed by reactions well known in the art.

Detailed Experimental Procedure of Synthesis, Specific Embodiments

Ethyl 4-iodobenzoate (Compound A)

To a suspension of 24.9 g (100.4 mmol) of 4-iodobenzoic acid in 46.25 g (58.9 ml, 1.0 mol) of ethanol (95%) was added 3.0 ml of conc. sulfuric acid. The resulting mixture was refluxed for 60 minutes, and then distilled until a clear, homogeneous solution was obtained. The solution was allowed to cool to room temperature, partitioned between 250 ml of water and 250 ml of pentane, and the layers were separated. The aqueous phase was washed with 3.times.100 ml-portions of pentane. All organic phases were combined, washed with brine solution, dried over MgSO.sub.4, filtered and concentrated in vacuo to a dark yellow oil. Purification by flash chromatography (silica, 10% ethyl acetate in hexane) yielded the title compound as a clear, light yellow oil. PMR (CDCl.sub.3): d 1.39 (3H, t, J=7.2 Hz), 4.37 (2H, q, J=7.2 Hz), 7.73-7.82 (4H, m).

6-Iodonicotinic acid (Compound B)

To 27.97 g (186.6 mmol) of sodium iodide cooled to -78.degree. C. was added 121.77 g (71.6 ml, 952.0 mmol) of hydriodic acid (in 57 wt % aqueous solution). The reaction mixture was allowed to warm slightly with stirring for 5 minutes, and then 30 g (190.4 mmol) of 6-chloronicotinic acid was added. The resulting mixture was allowed to warm to room temperature with stirring and then heated at 120-125.degree. C. in an oil bath for 42 hours. A dark brown layer formed above the yellow solid material. The reaction mixture was allowed to cool to room temperature and then poured into acetone (chilled to 0.degree. C.). The resultant yellow solid was collected by filtration, washed with 200 ml of 1N NaHSO.sub.3 solution, and dried in vacuum (3 mm Hg) to give the title compound as a pale yellow solid. PMR (DMSO-d.sub.6): d 7.90 (1H, dd, J=8.1, 2 Hz), 7.99 (1H, d, J=8.1 Hz), 8.80 (1H, d, J=2 Hz).

Ethyl 6-iodonicotinate (Compound C)

To a suspension of 23.38 g (94.2 mmol) of 6-iodonicotinic acid in 100 ml of dichloromethane was added a solution of 19.86 g (103.6 mmol) of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride in 250 ml of dichloromethane. To this suspension was added 12.40 g (15.8 ml, 269.3 mmol) of ethanol (95%) and 1.15 g (9.4 mmol) of 4-dimethylaminopyridine. The resulting solution was then heated at 50.degree. C. in an oil bath for 24.5 hours, concentrated in vacuo, partitioned between 200 ml of water and 250 ml of ethyl ether, and the layers were separated. The aqueous phase was washed with 2.times.150 ml-portions of ethyl ether. All organic phases were combined, washed

once with 75 ml of brine solution, dried over MgSO.sub.4, filtered and concentrated in vacuo to yield a yellow solid residue. Purification by flash chromatography (silica, 10% ethyl acetate in hexane) yielded the title compound as a white solid. PMR (CDCl.sub.3): d 1.41 (3H, t, J=7.1 Hz), 4.41 (2H, q, J=7.1 Hz), 7.85 (1H, d, J=8.2 Hz), 7.91 (1H, dd, J=8.2, 2.1 Hz), 8.94 (1H, d, J=2.1 Hz).

5-Bromo-2-t-butylphenol (Compound D)

To a solution of 29.46 g (170.3 mmol) of 3-bromophenol (distilled) and 16.41 g (20.9 ml, 221.3 mmol) of t-butanol in 100 ml of carbon tetrachloride was added 20 ml of conc. sulfuric acid. The clear, colorless solution turned a dark magenta color and became hot. The solution was cooled in water (ambient temperature) and allowed to stir at room temperature for 84 hours. The reaction mixture was neutralized with sat. NaHCO.sub.3 solution (pH .about.7.0), partitioned between 300 ml of water and 500 ml of dichloromethane, and the organic and aqueous layers were separated. The aqueous phase was washed with 2.times.500 ml-portions of dichloromethane. All organic phases were combined, washed with 400 ml of brine solution, dried over MgSO.sub.4, filtered and concentrated in vacuo to a purple oil. Purification by flash chromatography (silica, 5% ethyl acetate in hexane) followed by kugelrohr distillation (85-95.degree. C., 2 mm Hg) yielded the title compound as a clear, slightly yellow oil.

PMR (CDCl.sub.3): d 1.37 (9H, s), 4.89 (1H, s), 6.83 (1H, d, J=2.0 Hz), 6.99 (1H, dd, J=8.5, 2.0 Hz), 7.12 (1H, d, J=8.5 Hz).

4-Bromo-2-t-butylphenol (Compound E)

Using the same procedure as for the preparation of 5-bromo-2-t-butylphenol (Compound D), but instead using 50 g (289.0 mmol) of 4-bromophenol, 21.40 g (27.25 ml, 289.0 mmol) of t-butanol and 14 ml of conc. sulfuric acid (added slowly) and 140 ml of distilled carbon tetrachloride, stirred at room temperature for 24 hours produced a dull green-colored solution and a white precipitate. At this time an additional 3.5 ml of conc. sulfuric acid was added and the solution was allowed to stir for 4 days more at room temperature. After aqueous workup, a dark yellow oil was isolated. Purification by flash chromatography (silica, 2% ethyl acetate in hexane) yielded the title compound as a clear, light yellow oil. PMR (CDCl.sub.3): d 1.38 (9H, s), 4.79 (1H, s), 6.54 (1H, d, J=8.5 Hz), 7.16 (1H, dd, J=8.5, 2.5 Hz), 7.35 (1H, d, J=2.5 Hz).

4-Bromo-2-t-butyl-1-(2-tetrahydropyranoxy)benzene (Compound H)

To a solution of 18.92 g (82.6 mmol) of 4-bromo-2-t-butylphenol (Compound E) in 110 ml of dichloromethane was added dropwise 10.42 g (11.3 ml, 123.9 mmol) of 3,4-dihydro-2H-pyran. To this clear, colorless solution was added 1.86 g (7.4 mmol) of pyridinium p-toluenesulfonate. The resulting mixture was stirred at room temperature under a blanket of argon for 26.5 hours, partitioned between 250 ml of water and 400 ml of hexane, and the layers were separated. The organic phase was washed with 2.times.250 ml-portions of water, washed once with 150 ml of brine solution, dried over MgSO.sub.4, filtered and concentrated in vacuo to a yellow oil. Purification by flash chromatography (silica, 5% ethyl acetate in hexane) yielded the title compound as a light yellow, crystalline solid. PMR (CDCl.sub.3): d 1.39 (9H, s), 1.6-2.1 (6H, m), 3.6-3.7 (1H, m), 3.8-3.9 (1H, m), 5.43 (1H, t, J=2.7 Hz), 7.06 (1H, d, J=8.8 Hz), 7.24 (1H, dd, J=8.8, 2.7 Hz), 7.36 (1H, d, J=2.7 Hz).

5-Bromo-2-t-butyl-1-(2-tetrahydropyranoxy)benzene (Compound I)

Using the same procedure as for the preparation of 4-bromo-2-t-butyl-1-(2-tetrahydropyranoxy)benzene (Compound H), but instead using 8.18 g (35.7 mmol) of 5-bromo-2-t-butylphenol (Compound D), 0.90 g, 3.6 mmol) of pyridinium p-toluenesulfonate and 4.50 g (4.9 ml, 53.6 mmol) of 3,4-dihydro-2H-pyran and 50 ml of dichloromethane stirred at room temperature for 21 hours produced a dark yellow solution. At this time an additional 1.80 g (7.2 mmol) of pyridinium p-toluenesulfonate was added to the solution and it was allowed to stir at room temperature for 19 hours. After aqueous workup a clear, yellow oil was isolated. Purification by flash chromatography (silica, 5% ethyl acetate in hexane) yielded the title compound as a clear, slightly yellow oil. PMR (CDCl.sub.3): d 1.38 (9H, s), 1.6-2.1 (6H, m), 3.65-3.75 (1H, m), 3.8-3.95 (1H, m), 5.45 (1H, t, J=2.5 Hz), 7.03 (1H, dd, J=8.4, 2.0 Hz), 7.13 (1H, d, J=8.4 Hz), 7.33 (1H, d, J=2.0 Hz).

2-[[2-t-Butyl-1-(2-tetrahydropyranoxy)]-4-phenyl]acetylene (Compound N)

A sealed tube was purged under a slight vacuum with a stream of argon gas for several minutes. To this tube was added 20 ml of triethylamine (distilled over solid KOH). Under slight vacuum, the solvent was degassed with a stream of argon gas for 2 minutes and then 5 g (16 mmol) of 4-bromo-2-t-butyl-1-(2-tetrahydropyranoxy)benzene (Compound H) and 0.61 g (3.2 mmol) of cuprous iodide (ground to a powder) were added. The resulting yellow mixture was degassed (as described above) for 3.5 minutes. To the degassed mixture was added 2.33 g (3.3 mmol) of bis(triphenyl)phosphine palladium (II) chloride and 15 ml of triethylamine. The reaction mixture was degassed for 5.5 minutes and then 7.71 g (11.1 ml, 78.5 mmol) of trimethylsilyl acetylene was added. The tube was sealed and then heated in an oil bath at 55.degree. C. for 3 days. The solution turned dark brown and a black solid formed. The solid was filtered over celite, washed with approximately 350 ml of ethyl ether and discarded. The filtrate was washed with 3.times.150 ml-portions of water, washed once with 100 ml of brine solution, dried over K.sub.2 CO.sub.3, filtered and concentrated in vacuo to a dark brown, viscous oil. Purification by flash chromatography (silica, 1% ethyl acetate in hexane) yielded 2-[2-t-butyl-1-(2-tetrahydropyranoxy)-4-phenyl]-1-trimethylsilyl acetylene. The crude TMS-acetylene was dissolved in 35 ml of methanol and 0.16 g (1.2 mmol) of anhydrous potassium carbonate was added to the solution. The solution was allowed to stir at room temperature overnight, concentrated in vacuo, diluted with 35 ml of sat. NaHCO.sub.3 solution and allowed to stir at room temperature for 5 minutes. The solution was extracted with 50 ml of dichloromethane and the layers were separated. The aqueous layer was washed with 3.times.50 ml-portions of dichloromethane. All organic phases were combined, washed once with 100 ml of water, washed once with 50 ml of brine solution, dried over MgSO.sub.4, filtered and concentrated in vacuo to yield an orange solid. Purification by flash chromatography (silica, 5% ethyl acetate in hexane) yielded the title compound as a yellow solid. PMR (CDCl.sub.3): d 1.40 (9H, s), 1.6-2.1 (6H, m), 2.98 (1H, s), 3.6-3.7 (1H, m), 3.8-3.9 (1H, m), 5.49 (1H, t, J=2.6 Hz), 7.11 (1H, d, J=8.5 Hz), 7.31 (1H, dd, J=8.5, 2.1 Hz), 7.43 (1H, d, J=2.1 Hz).

2-[[2-t-Butyl-1-(2-tetrahydropyranoxy)]-5-phenyl]acetylene (Compound O)

Using the same procedure as for the preparation of 2-[[2-t-butyl-1-(2-tetrahydropyranoxy)]-4-phenyl]acetylene (Compound N), but instead using 10.07 g (32.1 mmol) of 5-bromo-2-t-butyl-1-(2-tetrahydropyranoxy)benzene (Compound I), 1.53 g (8.0 mmol) of cuprous iodide (ground to a powder), 5.64 g (8.0 mmol) of bis(triphenyl)phosphine palladium (II) chloride, 15.79 g (22.7 ml, 160.7 mmol) of trimethylsilyl acetylene and 70 ml of diethylamine (distilled over solid KOH) heated in an oil bath at 55.degree. C. for 43.5 hours produced a brown solution. At this time, an additional 0.78 g (4.1 mmol) of cuprous iodide (ground to a powder), 2.82 g (4.0 mmol) of palladium (II) catalyst and 10.4 ml (73.6 mmol) of TMS-acetylene were added to the mixture. The tube was resealed and heated at 55.degree. C. in an oil bath for 2 days to give a brown oil following aqueous workup. Purification by flash chromatography (pre-absorbed onto silica with chloroform, eluted with 5% ethyl acetate in hexane) yielded 2-[[2-t-butyl-1-(2-tetrahydropyranoxy)]-5-phenyl]-1-trimethylsilyl acetylene. The crude TMS-acetylene was converted into the title compound by using 0.78 g (5.7 mmol) of anhydrous potassium carbonate and 100 ml of methanol to give a brown oil following aqueous workup. Purification by flash chromatography (silica, 3% ethyl acetate in hexane) yielded the title compound as an orange oil. PMR (CDCl.sub.3): d 1.40 (9H, s), 1.6-2.1 (6H, m), 3.01 (3H, s), 3.6-3.7 (1H, m), 3.8-3.95 (1H, m), 5.47 (1H, t, J=2.7 Hz), 7.06 (1H, dd, J=8.0, 1.7 Hz), 7.22 (1H, d, J=8.0 Hz), 7.31 (1H, d, J=1.7 Hz).

Ethyl 4-[2-[[2-t-butyl-1-(2-tetrahydropyranoxy)]-4-phenyl]-ethyn-1-yl]benzoate (Compound 1)

To a 100 ml 3-necked round bottom flask (fitted with a glass stopper, reflux condenser, and a rubber septum) was added 25 ml of diethylamine (distilled over solid KOH). The solvent was degassed with a vigorous stream of argon gas for several minutes. To this solution was added 2.67 g (10.3 mmol) of 2-[[2-t-butyl-1-(2-tetrahydropyranoxy)]-4-phenyl]acetylene (Compound N) dissolved in 10 ml of diethylamine, 0.39 g (2.1 mmol) of cuprous iodide (ground to a powder), and 2.72 g (9.8 mmol) of ethyl 4-iodobenzoate (Compound A) dissolved in 5 ml of diethylamine. The resultant yellow solution was degassed for 10 minutes after which 1.67 g (2.4 mmol) of bis(triphenyl)phosphine palladium (II) chloride was added. The solution was cooled to 0.degree. C., degassed for 5 minutes, and then stirred at 0.degree. C. for 25 minutes. The reaction mixture was allowed to warm to room temperature and then stirred overnight. A salt formed against the walls of the flask. The reaction mixture was filtered through celite, washed with 500 ml of ethyl ether and the celite discarded. The filtrate was extracted with

200 ml of water and the layers were separated. The organic phase was washed with 3.times.200 ml-portions of water, washed once with 150 ml of brine solution, dried over K.sub.2 CO.sub.3, filtered and concentrated in vacuo to yield a yellow foam. Purification by flash chromatography (silica, 5% ethyl acetate in hexane) followed by recrystallization from boiling methanol yielded the title compound as beige needles. PMR (CDCl.sub.3): d 1.38 (3H, t, J=7.1 Hz), 1.43 (9H, s), 1.6-2.1 (6H, m), 3.6-3.7 (1H, m), 3.8-3.95 (1H, m), 4.38 (2H, q, J=7.1 Hz), 5.52 (1H, t, J=2.5 Hz), 7.16 (1H, d, J=8.5 Hz), 7.36 (1H, dd, J=8.5, 2.1 Hz), 7.48 (1H, d, J=2.1 Hz), 7.57 (2H, d, J=8.4 Hz), 8.01 (2H, d, J=8.4 Hz).

4-[2-[[2-t-Butyl-1-(2-tetrahydropyranoxy)]-4-phenyl]ethyn-1-yl]benzoic acid (Compound 2)

To a solution of 2.00 g (4.9 mmol) of ethyl 4-[2-[[2-t-butyl-1-(2-tetrahydropyranoxy)]-4-phenyl]ethyn-1-yl]-benzoate (Compound 1) in 80 ml of tetrahydrofuran was added 19.7 ml (9.8 mmol) of a 0.5 M LiOH solution. The yellow, homogeneous solution was allowed to stir at room temperature for 19 hours. The reaction mixture was concentrated in vacuo, partitioned between 100 ml of water and 60 ml of hexane and the layers were separated. The aqueous phase was diluted with 200 ml of ethyl ether, cooled to 0.degree. C. and acidified with 1 N sulfuric acid to an approximate pH of 4-5. The layers were separated and the aqueous layer was discarded. The organic phase was washed once with brine solution, dried over MgSO.sub.4, filtered and concentrated in vacuo to yield a white solid. The solid was recrystallized from boiling acetonitrile to give the title compound as fine, white needles. PMR (CDCl.sub.3): d 1.43 (9H, s), 1.6-2.1 (6H, m), 3.6-3.75 (1H, m), 3.8-3.95 (1H, m), 5.52 (1H, br s), 7.17 (1H, d, J=8.6 Hz), 7.37 (1H, dd, J=8.6, 2.0 Hz), 7.48 (1H, d, J=2.0 Hz), 7.60 (2H, d, J=8.6 Hz), 8.07 (2H, J=8.6 Hz).

Ethyl 6-[2-[[2-t-butyl-1-(2-tetrahydropyranoxy)]-5-phenyl]-ethyn-1-yl]nicotinoat e (Compound 5)

Using the same procedure as for the preparation of ethyl 4-[2-[[2-t-butyl-1-(2-tetrahydropyranoxy)]-4-phenyl]-ethyn-1-yl]benzoate (Compound 1), but instead using 2.21 g (8.6 mmol) of 2-[[2-t-butyl-1-(2-tetrahydropyranoxy)]-5-phenyl]acetylene (Compound 0), 0.45 g (2.4 mmol) of cuprous iodide (ground to a powder), 2.15 g (7.8 mmol) of ethyl 6-iodonicotinate (Compound C), 1.89 g (2.7 mmol) of bis(triphenyl)phosphine palladium (II) chloride and 45 ml of diethylamine stirred at room temperature overnight (27.5 hours) gave an orange foam. Purification by flash chromatography (pre-absorbed onto silica with chloroform, eluted with 10% ethyl acetate in hexane) followed by recrystallization from boiling methanol yielded the title compound as bright yellow, needles. PMR (CDCl.sub.3): d 1.42 (3H, t, J=7 Hz), 1.42 (9H, s), 1.6-2.1 (6H, m), 3.65-3.8 (1H, m), 3.85-3.95 (1H, m), 4.43 (2H, q, J=7 Hz), 5.50 (1H, t, J=2.4 Hz), 7.21 (1H, dd, J=8.1, 1.7 Hz), 7.29 (1H, d, J=8.1 Hz), 7.44 (1H, d, J=1.7 Hz), 7.60 (1H, d, J=8.2 Hz), 8.29 (1H, dd, J=8.2, 2.2 Hz), 9.20 (1H, d, J=2.2 Hz).

Ethyl 4-[2-[[2-t-butyl-1-(2-tetrahydropyranoxy)]-5-phenyl]-ethyn-1-yl]benzoate (Compound 6)

Using the same procedure as for the preparation of ethyl 4-[2-[[2-t-butyl-1-(2-tetrahydropyranoxy)]-4-phenyl]-ethyn-1-yl]benzoate (Compound 1), but instead using 3.30 g (12.8 mmol) of 2-[[2-t-butyl-1-(2-tetrahydropyranoxy)]-5-phenyl]acetylene (Compound 0), 0.44 g (2.3 mmol) of cuprous iodide (ground to a powder), 3.20 g (11.6 mmol) of ethyl 4-iodobenzoate (Compound A), 1.87 g (2.7 mmol) of bis(triphenyl)phosphine palladium (II) chloride and 50 ml of diethylamine gave, after aqueous work up, an orange foam. Purification by flash chromatography (preabsorbed onto silica with chloroform, eluted with 5% ethyl acetate in hexane) followed by recrystallization from boiling methanol yielded the title compound as light brown, clusters. PMR (CDCl.sub.3): d 1.40 (3H, t, J=7.1 Hz), 1.42 (9H, s), 1.6-2.1 (6H, m), 3.6-3.75 (1H, m), 3.85-3.95 (1H, m), 4.38 (2H, q, J=7.1 Hz), 5.53 (1H, br s), 7.11 (1H, dd, J=8.1, 2 Hz), 7.27 (1H, d, J=8.1 Hz), 7.36 (1H, d, J=2 Hz), 7.57 (2H, d, J=8.4 Hz), 8.01 (2H, d, J=8.4 Hz).

4-[2-[[2-t-Butyl-1-(2-tetrahydropyranoxy)]-5-phenyl]ethyn-1-yl]-1-benzoi c acid (Compound 7)

Using the same procedure as for the preparation of 4-[2-[[2-t-butyl-1-(2-tetrahydropyranoxy)]-4-phenyl]ethyn-1-yl]benzoic acid (Compound 2), but instead using 2.01 g (5.1 mmol) of ethyl 4-[2-[[2-t-butyl-1-(2-tetrahydropyranoxy)]-5-phenyl]- ethyn-1-yl]benzoate (Compound 6), 10.5 ml (10.5 mmol) of 1.0 M LiOH solution and 44 ml of

tetrahydrofuran (THF), the resulting solution was allowed to stir at room temperature for 48 hours. Thereafter it was refluxed overnight to give, after aqueous work up, a white solid. Purification by flash chromatography (silica, 10% ethyl acetate in hexane followed by 15% methanol in dichloromethane) yielded the title compound as an off-white solid. PMR (DMSO-d.sub.6): d 1.39 (9H, s), 1.55-2.0 (6H, m), 3.6-3.8 (2H, m), 5.64 (1H, t,), 7.14 (1H, dd, J=8.0, 1.6 Hz), 7.26 (1H, d, J=1.6 Hz), 7.30 (1H, d, J=8.0 Hz), 7.62 (2H, d, J=8.3 Hz), 7.97 (2H, d, J=8.3 Hz).

6-[2-[[2-t-Butyl-1-(2-tetrahydropyranoxy)]-5-phenyl]ethyn-1-yl]nicotinic acid (Compound 8)

Using the same procedure as for the preparation of 4-[2-[[2-t-butyl-1-(2-tetrahydropyranoxy)]-4-phenyl]ethyn-1-yl]benzoic acid (Compound 2), but instead using 1.50 g (3.8 mmol) of ethyl 6-[2-[[2-t-butyl-1-(2-tetrahydropyranoxy)]-5-phenyl]ethyn-1-yl]nicotinoate (Compound 5), 8.0 ml (8.0 mmol) of a 1.0 M LiOH solution and 32 ml of tetrahydrofuran stirred at room temperature for 48 hours gave, after aqueous work up, a yellow solid. The solid was recrystallized from boiling acetonitrile to give the title compound as bright yellow crystals. PMR (DMSO-d.sub.6): d 1.40 (9H, s), 1.55-2.0 (6H, m), 3.6-3.8 (2H, m), 5.65 (1H, br s), 7.21 (1H, dd, J=8, 1.7 Hz), 7.3-7.35 (2H, m), 7.77 (1H, d, J=8.2 Hz), 8.29 (1H, dd, J=8.2, 2.2 Hz), 9.06 (1H, d, J=2.2 Hz).

Ethyl (3-bromophenyl)acetate (Compound B.sub.1)

100 g (463 mmol) of 3-bromophenylacetic acid was converted into the title compound (yellow oil) using 2 g of conc. H.sub.2 SO.sub.4 and 500 ml of ethanol by refluxing the reaction for 16 hours. Thereafter, the reaction mixture was cooled to ambient temperature, stirred with solid K.sub.2 CO.sub.3 for 30 minutes and then filtered. The filtrate was concentrated in vacuo, diluted with Et.sub.2 O, washed with 10% aqueous NaHCO.sub.3 and brine, dried over MgSO.sub.4 and concentrated in vacuo to give the title compound.

PMR (CDCl.sub.3): .delta. 1.26 (3H, t, J=7.0 Hz), 3.56 (2H, s), 4.16 (2H, q, J=7.0 Hz), 7.16-7.26 (2H, m), 7.38-7.46 (2H, m).

Ethyl 4-(3-bromophenyl)butanoate (Compound D.sub.1)

60 g (246 mmol) of ethyl(3-bromophenyl)acetate (Compound B.sub.1) was converted into the title compound (oil) using 255 ml (255 mmol) of diisobutyl aluminum hydride (DIBAL-H, 1M in hexane), 85.8 g (250 mmol) of (carbethoxy methylene)triphenylphosphorane and 1.7 g of 10% Pd/C. The procedure was as follows: To a cold solution (-78.degree. C.) of Compound B.sub.1 in CH.sub.2 Cl.sub.2 was added dropwise (over a span of 1 hour) the diisobutyl aluminum hydride (DIBAL-H, 1M solution in hexane). After the DIBAL-H addition was complete, the reaction was stirred at -78 .degree. C. for an additional hour. The reaction was quenched by the dropwise addition of methanol, followed by water and 10% HCl. The mixture was then warmed to 0.degree. C., stirred for 10 minutes and then washed with water, 10% aqueous NaHCO.sub.3 and brine. The organic phase was dried over MgSO.sub.4 and the solvent distilled off at ambient temperature to give crude (3-bromophenyl)acetaldehyde. To a cold solution (0.degree. C.) of this crude aldehyde in CH.sub.2 Cl.sub.2 was added a solution of the (carbethoxy methylene)triphenylphosphorane reagent in CH.sub.2 Cl.sub.2. The mixture was stirred for 16 hours, concentrated in vacuo and purified by flash chromatography (silica, 10% EtOAc-hexane) to give ethyl 4-(3-bromophenyl)but-2-enoate as a mixture of E:Z isomers. This isomeric mixture was dissolved in EtOAc and hydrogenated over 10% Pd/C for 6 hours. The catalyst was filtered off and the filtrate concentrated in vacuo to give the title compound as a white solid.

PMR (CDCl.sub.3): .delta. 1.26 (3H, t, J=7.1 Hz), 1.89-2.00 (2H, m), 2.31 (2H, t, J=7.5 Hz), 2.63 (2H, t, J=7.2 Hz), 4.15 (2H, q, J=7.1 Hz), 7.10-7.35 (4H, m).

5-(3-bromophenyl)-2-methylpentan-2-ol (Compound E.sub.1)

To a cold solution (0.degree. C.) of 17 g (63 mmol) of ethyl 4-(3-bromophenyl)butanoate (Compound D.sub.1) in 40 ml of THF was added 63 ml (189 mmol) of methylmagnesium bromide (3.0M solution in THF). The reaction was stirred at 0.degree. C. for 2 hours, quenched by the slow addition of ice cold water (30 ml) followed by 10% HCl (30 ml) and then extracted with Et.sub.2 O (4.times.60 ml). The combined organic layer was washed with 10% aqueuos NaHCO.sub.3 (10 ml), water (10 ml) and brine (10 ml), dried over MgSO.sub.4 and concentrated in vacuo. Purification by kugelrohr distillation gave the title compound as a colorless oil.

PMR (CDCl.sub.3): .delta. 1.20 (6H, s), 1.43-1.55 (2H, m), 1.62-1.78 (2H, m), 2.60 (2H, t, J=6.0 Hz), 7.10-7.41 (4H, m).

6-Bromo-1,2,3,4-tetrahydro-1,1-dimethylnaphthalene (Compound F.sub.1)

15.0 g (58.3 mmol) of 5-(3-bromophenyl)-2-methylpentan-2-ol (Compound E.sub.1) was cooled to 0.degree. C. and then 2.8 ml of conc. H.sub.2 SO.sub.4 was added. The mixture was stirred for 2.5 hours, diluted with water (20 ml) and extracted with Et.sub.2 O (3.times.40 ml). The combined organic layers were washed with water, sat. aqueous NaHCO.sub.3 and brine, dried over MgSO.sub.4 and concentrated in vacuo. Purification by kugelrohr distillation gave the title compound as a colorless oil.

PMR (CDCl.sub.3): .delta. 1.25 (6H, s), 1.61-1.66 (2H, m), 1.74-1.82 (2H, m), 2.73 (2H, t, J=6.0 Hz), 7.16-7.26 (3H, m).

7-Bromo-3,4-dihydro-4,4-dimethyl-naphthalen-1(2H)-one (Compound G.sub.1)

To a cold mixture (0.degree. C.) of 209 g (200 mmol) of chromium trioxide, 100 ml (1.06 mol) of acetic anhydride and 200 ml (3.5 mol) of acetic acid was added a solution of 10 g (41.8 mmol) of 6-bromo-1,2,3,4-tetrahydro-1,1-dimethylnaphthalene (Compound F.sub.1) in 125 ml of benzene. The reaction mixture was stirred for 1 hour, quenched with ice cold water and extracted with Et.sub.2 O (3.times.100 ml). The organic layer was dried over MgSO.sub.4, concentrated in vacuo, and purified by column chromatography (silica, 10% EtOAc-hexane) to give the title compound as a white solid.

PMR (CDCl.sub.3): .delta. 1.28 (6H, s), 2.01 (2H, t, J=6.0 Hz), 2.72 (2H, t, J=6.0 Hz), 7.31 (1H, d, J=9.0 Hz), 7.61 (1H, dd, J=3.0, 9.0 Hz), 8.11 (1H, d, J=3.0 Hz).

7-Ethynyl-3,4-dihydro-4,4-dimethyl-naphthalen-1(2H)-one (Compound L.sub.1)

7 g (27.6 mmol) of 7-bromo-3,4-dihydro-4,4-dimethyl-naphthalen-1(2H)-one (Compound G.sub.1) was converted into the title compound using 39 ml (36.6 mmol) of trimethylsilyl acetylene, 0.97 g (1.3 mmol) of bis(triphenylphosphine)palladium(II) chloride, 0.26 g (1.3 mmol) of cuprous iodide and 0.6 g (4.3 mmol) of K.sub.2 CO.sub.3. The procedure was as follows: To a solution, flushed for 15 minutes with a stream of argon, of Compound G.sub.1 in triethylamine was added the bis(triphenylphosphine)palladium(II) chloride and cuprous iodide. The solution mixture was flushed with argon for 5 minutes and then the trimethylsilyl acetylene was added. The reaction mixture was sealed in a pressure tube and placed in a preheated oil bath (100.degree. C.) for 24 hours. The reaction mixture was then filtered through celite, washed with Et.sub.2 O and the filtrate concentrated in vacuo to give crude 7-(2-trimethylsilyl)ethynyl-3,4-dihydro-4,4-dimethyl-naphthalen-1(2H)-one. To a solution of this crude TMS-acetylenic compound in methanol was added K.sub.2 CO.sub.3. The mixture was stirred for 8 hours at ambient temperature and then filtered. The filtrate was concentrated in vacuo, diluted with Et.sub.2 O, washed with water, 10% HCl and brine, dried over MgSO.sub.4 and concentrated in vacuo. Purification by column chromatography (silica, 10% EtOAc-hexane) yielded the title compound as a white solid.

PMR (CDCl.sub.3): .delta. 1.39 (6H, s), 2.02 (2H, t, J=7.0 Hz), 2.73 (2H, t, J=7.0 Hz), 3.08 (1H, s), 7.39 (1H, d, J=8.2 Hz), 7.61 (1H, dd, J=1.8 , 8.2 Hz), 8.14 (1H, d, J=9 1.8 Hz).

Ethyl 4-[(5,6,7,8-tetrahydro-8,8-dimethyl-5-oxonaphth-3-yl)ethynyl]benzoate (Compound 9)

4 g (21.7 mmol) of 7-ethynyl-3,4-dihydro-4,4-dimethyl-naphthalen-1(2H)-one (Compound L.sub.1) was converted into the title compound using 6 g (21.7 mmol) of ethyl 4-iodobenzoate, 5 g (7.2 mmol) of bis(triphenylphosphine)palladium(II) chloride and 1.4 g (7.2 mmol) of cuprous iodide. The procedure was as follows: To a solution of Compound L.sub.1, flushed for 15 minutes with a stream of argon, and ethyl 4-iodobenzoate in triethylamine was added the bis(triphenylphosphine)palladium(II) chloride catalyst and the cuprous iodide. The solution mixture was flushed with argon for 5 minutes and then stirred at ambient temperature for 18 hours. The reaction mixture was filtered through celite and the filtrate concentrated in vacuo. Purification by flash chromatography (silica, 10% EtOAc-hexane) yielded the title compound as a white solid.

PMR (CDCl.sub.3): .delta. 1.41 (3H, t, J=7.2 Hz), 1.41 (6H, s), 2.04 (2H, t, J=6.5 Hz), 2.76 (2H, t, J=6.5 Hz), 4.40 (2H, q, J=7.2 Hz), 7.44 (1H, d, J=8.2 Hz), 7.59 (2H, d, J=8.4 Hz), 7.68 (1H, dd, J=1.8, 8.2 Hz), 8.04 (2H, d, J=8.4 Hz), 8.15 (1H, d, J=1.8 Hz).

Ethyl 4-[(5,6,7,8-tetrahydro-5-hydroxy-8,8-dimethylnaphth-3-yl)ethynyl]benzoate (Compound 10)

1 g (2.88 mmol) of ethyl 4-[(5,6,7,8-tetrahydro-8,8-dimethyl-5-oxonaphth-3-yl)ethynyl]benzoate (Compound 9) was converted into the title compound using 60 mg (1.6 mmol) of sodium borohydride. The procedure was as follows: To a cold solution (0.degree. C.) of Compound 9 in 5 ml of THF and 10 ml of ethanol was added sodium borohydride. The mixture was stirred for 6 hours, diluted with water (10 ml) and extracted with Et.sub.2 O (4.times.40 ml). The combined organic layers were washed with 10% HCl (5 ml), 10% aqueous NaHCO.sub.3 (10 ml) and brine (10 ml), dried over MgSo.sub.4 and concentrated in vacuo to give the title compound as a white solid.

PMR (CDCl.sub.3): .delta. 1.26 (3H, s), 1.33 (3H, s), 1.40 (3H, t, J=7.1 Hz), 1.58-1.70 (1H, m), 1.80-1.95 (2H, m), 2.04-2.14 (1H, m), 4.38 (2H, q, J=7.1 Hz), 4.72 (1H, q, J=5.1 Hz), 7.32 (1H, d, J=8.2 Hz), 7.41 (1H, dd, J=1.8, 8.2 Hz), 7.56 (2H, d, J=8.5 Hz), 7.65 (1H, d, J=1.8 Hz), 8.01 (2H, d, J=8.5 Hz).

Ethyl 4-[[5,6,7,8-tetrahydro-5(RS)-(2'(RS)-tetrahydropyranyloxy)-8,8-dimethylnaphth-3-yl]ethynyl]benzoate (Compound 11) and Ethyl 4-[[5,6,7,8-tetrahydro-5(RS)-(2'(SR)-tetrahydropyranyloxy)-8,8-dimethylnaphth-3-yl]ethynyl]benzoate (Compound 12)

500 mg (1.44 mmol) of ethyl 4-[(5,6,7,8-tetrahydro-5-hydroxy-8,8-dimethylnaphth-3-yl)ethynyl]benzoate (Compound 10) was converted to a mixture of diastereomers using 400 mg (4.8 mmol) of 3,4-dihydro-2H-pyran and 50 mg (0.2 mmol) of pyridinium p-toluenesulfonate. The procedure was as follows: To a cold solution (0.degree. C.) of Compound 10 in CH.sub.2 Cl.sub.2 was added 3,4-dihydro-2H-pyran (DHP) followed by the pyridinium p-toluenesulfonate (PPTS) catalyst. The reaction mixture was stirred at ambient temperature for 16 hours and then 1 g of K.sub.2 CO.sub.3 was added. The mixture was stirred for 5 minutes, washed with water and brine, dried over MgSO.sub.4 and concentrated in vacuo to a gummy mixture of two diastereomers. HPLC separation (Partisil 10, 10% EtOAc-hexane) of the diastereomers gave the title compounds (RT=65 and 70 minutes), respectively.

Ethyl 4-[[5,6,7,8-tetrahydro-5(RS)-(2'(RS)-tetrahydropyranyloxy)-8,8-dimethylnaphth-3-yl]ethynyl]benzoate (Compound 11)

PMR (CDCl.sub.3): (RT=65 minutes) .delta. 1.26 (3H,s), 1.32 (3H,s), 1.43 (3H, t, J=7.1 Hz), 1.51-2.20 (10H, m), 3.55-3.62 (1H, m), 3.95-4.05 (1H, m), 4.39 (2H, q, J=7.1 Hz), 4.64 (1H, t, J=5.9 Hz), 4.89 (1H, t, J=2.9 Hz), 7.33 (1H, d, J=8.2 Hz), 7.41 (1H, dd, J=1.8, 8.2 Hz), 7.46 (1H, d, J=1.8 Hz), 7.57 (2H, d, J=8.2 Hz), 8.01 (2H, d, J=8.2 Hz).

Ethyl 4-[[5,6,7,8-tetrahydro-5(RS)-(2'(SR)-tetrahydropyranyloxy)-8,8-dimethylnaphth-3-yl]ethynyl]benzoate (Compound 12)

PMR (CDCl.sub.3): (RT=70 minutes) .delta. 1.26 (3H,s), 1.32 (3H,s), 1.40 (3H, t, J=7.1 Hz), 1.52-1.68 (5H, m), 1.72-1.95 (4H, m), 1.96-2.10 (1H, m), 3.55-3.65 (1H, m), 4.00-4.10 (1H, m), 4.38 (2H, q, J=7.1 Hz), 4.77 (1H, t, J=6.1 Hz), 4.89 (1H, t, J=2.5 Hz), 7.30 (1H, d, J=8.2 Hz), 7.40 (1H, dd, J=1.8, 8.2 Hz), 7.57 (2H, d, J=8.5 Hz), 7.68 (1H, d, J=1.8 Hz),8.01 (2H, d, J=8.5 Hz).

4-[[5,6,7,8-tetrahydro-5(RS)-(2'(RS)-tetrahydropyranyloxy)-8,8-dimethyln aphth-3-yl]ethynyl]benzoic acid (Compound 13)

80 mg (0.19 mmol) of ethyl 4-[[5,6,7,8-tetrahydro-5(RS)-(2'(RS)-tetrahydropyranoxy)-8,8-dimethylnaphth-3-yl]ethynyl]benzoate (Compound 11) was converted into the title compound using 1 ml (1 mmol) of LiOH (1M aqueous solution). The procedure was as follows: To a solution of Compound 11 in 3 ml of THF and 1 ml of methanol was added LiOH (1M aqueous solution). The mixture was refluxed for 2 hours, cooled to ambient temperature, diluted with 100 ml of Et.sub.2 O:EtOAc (1:1, v/v) and acidified to pH 5 with ice-cold 10% HCl. The organic phase was washed with water (10 ml) and brine (10 ml), dried with MgSO.sub.4 and concentrated in vacuo to yield the title compound as a white solid.

PMR (CDCl.sub.3): .delta. 1.26 (3H, s), 1.33 (3H, s), 1.60-2.10 (10H, m),

3.55-3.65 (1H, m), 3.95-4.05 (1H, m), 4.65 (1H, t, J=5.5 Hz), 4.90 (1H, t, J=2.9 Hz), 7.34 (1H, d, J=8.2 Hz), 7.42 (1H, dd, J=1.8, 8.2 Hz), 7.46 (1H, d, J=1.8 Hz), 7.61 (2H, d, J=8.2 Hz), 8.07 (2H, d, J=8.2 Hz).

4-[[5,6,7,8-tetrahydro-5(RS)-(2'(SR)-tetrahydropyranyloxy)-8,8-dimethyln aph th-3-yl]ethynyl]benzoic acid (Compound 14)

Employing the same general procedure as for the preparation of 4-[[5,6,7,8-tetrahydro-5(RS)-(2'(RS)-tetrahydropyranyloxy)-8,8-dimethylnap hth-3-yl]ethynyl]benzoic acid (Compound 13), 100 mg (0.23 mmol) of ethyl 4-[[5,6,7,8-tetrahydro-5(RS)-(2'(SR)-tetrahydropyranoxy)-8,8-dimethylnapht h-3-yl]ethynyl]benzoate (Compound 12) was converted into the title compound using 0.6 ml (0.6 mmol) of LiOH (1M aqueous solution).

PMR (DMSO-d.sub.6): .delta. 1.22 (3H, s), 1.27 (3H, s), 1.40-1.60 (5H, m), 1.62-1.86 (4H, m), 1.90-2.05 (1H, m), 3.50-3.60 (1H, m), 3.87-4.00 (1H, m), 4.68 (1H, t, J=5.5 Hz), 4.80-4.85 (1H, m), 7.45 (2H, s), 7.54 (1H, s), 7.67 (2H, d, J=8.4 Hz), 7.97 (2H, d, J=8.4 Hz).

CLAIMS:

What is claimed is:

1. A method for treating a mammal, in need of such treatment, with a pharmaceutical composition containing as its active ingredient an effective amount of a compound capable of binding AP1 protein in a complex with said compound and with a retinoid receptor thereby inhibiting gene expression promoted by AP1 protein, said compound substantially unable to activate gene expression induced through binding of said compound to RAR.alpha. and RAR.GAMMA. retinoid receptors.

2. A method in accordance with claim 1 wherein in a transactivation assay utilized for measuring the ability of said compound to transactivate gene expression through RAR.alpha., RAR.beta. and RAR.GAMMA. receptors, the compound is at least 20 times less active through RAR.alpha. and through RAR.GAMMA. receptors than through RAR.beta. receptors.

3. A method for treating a mammal, in need of such treatment, with a pharmaceutical composition containing as its active ingredient an effective amount of a compound capable of binding AP1 protein in a complex with said compound and with a retinoid receptor thereby inhibiting gene expression promoted by AP1 protein, said compound substantially unable to activate gene expression induced through binding of said compound to RAR.alpha. and RAR.gamma. retinoid receptors where the active ingredient comprises a compound selected from formula 1 and formula 2 ##STR4## where X is N or CH, and R is H, or lower alkyl, or a pharmaceutically acceptable salt of said compound.

4. A method in accordance with claim 3 where R is H or a pharmaceutically acceptable salt thereof.

5. A method in accordance with claim 3 where R is C.sub.1 -C.sub.3 alkyl.

6. A method in accordance with claim 3 wherein the compound is in accordance with formula 1.

7. A method in accordance with claim 6 wherein the compound is selected from the group consisting of:

(1) 4-[2-[[2-t-butyl-1-(2-tetrahydropyranoxy)]-4-phenyl]ethyn-1-yl]benzoic acid;

(2) 4-[2-[[2-t-butyl-1-(2-tetrahydropyranoxy)]-5-phenyl]-ethyn-1-yl]-1-benzoic acid, and

(3) 6-[2-[[2-t-butyl-1-(2-tetrahydropyranoxy)]-5-phenyl]ethyn-1-yl]nicotinic a cid.

8. A method in accordance with claim 3 wherein the compound is in accordance with formula 2.

9. A method in accordance with claim 7 wherein the compound is selected from the group consisting of:

(1) 4-[[5,6,7,8-tetrahydro-5(RS)-(2'(RS)-tetrahydropyranyloxy)-8,8-dimethylnap

hth-3-yl]ethynyl]benzoic acid, and

(2) 4-[[5,6,7,8-tetrahydro-5(RS)-(2'(SR)-tetrahydropyranyloxy)-8,8-dimethylnap hth-3-yl]ethynyl]benzoic acid.

10. A method for repressing expression of AP1 protein responsive gene in a mammal in need of such repression for a therapeutic purpose, without significantly activating expression of genes which are activated through RAR.alpha. and RAR.GAMMA. retinoid receptors, said method comprising administering to said mammal a retinoid compound capable of binding AP1 protein in a complex with said compound and with a retinoid receptor thereby inhibiting gene expression promoted by AP1 protein, said compound substantially unable to activate gene expression promoted by RAR.alpha. and RAR.GAMMA. retinoid receptors.

11. A method in accordance with claim 10 wherein in a transactivation assay utilized for measuring the ability of said compound to transactivate gene expression through RAR.alpha., RAR.beta. and RAR.GAMMA. receptors, the compound is at least 20 times less active through RAR.alpha. and through RAR.GAMMA. receptors than through RAR.beta. receptors.

12. A method for repressing expression of AP1 protein responsive gene in a mammal in need of such repression for a therapeutic purpose, without significantly activating expression of genes which are activated through RAR.alpha. and RAR.gamma. retinoid receptors, said method comprising administering to said mammal a retinoid compound capable of binding AP1 protein in a complex with said compound and with a retinoid receptor thereby inhibiting gene expression promoted by AP1 protein, said compound substantially unable to activate gene expression promoted by RAR.alpha. and RAR.gamma. retinoid receptors where the active ingredient comprises a compound selected from formula 1 and formula 2 ##STR5## where X is N or CH, and R is H, or lower alkyl, or a pharmaceutically acceptable salt of said compound.

13. A method in accordance with claim 12 where R is H or a pharmaceutically acceptable salt thereof.

14. A method in accordance with claim 12 where R is C.sub.1 -C.sub.3 alkyl.

15. A method in accordance with claim 12 wherein the compound is in accordance with formula 1.

16. A method in accordance with claim 15 wherein the compound is selected from the group consisting of:

(1) 4-[2-[[2-t-butyl-1-(2-tetrahydropyranoxy)]-4-phenyl]ethyn-1-yl]benzoic acid;

(2) 4-[2-[[2-t-butyl-1-(2-tetrahydropyranoxy)]-5-phenyl]-ethyn-1-yl]-1-benzoic acid, and

(3) 6-[2-[[2-t-butyl-1-(2-tetrahydropyranoxy)]-5-phenyl]ethyn-1-yl]nicotinic a cid.

17. A method in accordance with claim 12 wherein the compound is in accordance with formula 2.

18. A method in accordance with claim 17 wherein the compound is selected from the group consisting of:

(1) 4-[[5,6,7,8-tetrahydro-5(RS)-(2'(RS)-tetrahydropyranyloxy)-8,8-dimethylnap hth-3-yl]ethynyl]benzoic acid, and

(2) 4-[[5,6,7,8-tetrahydro-5(RS)-(2'(SR)-tetrahydropyranyloxy)-8,8-dimethylnap hth-3-yl]ethynyl]benzoic acid.

Document Number 4

Entry 4 of 59 File: USPT Feb 15, 2000

DOCUMENT-IDENTIFIER: US 6025388 A
TITLE: Method for inhibiting gene expression promoted by AP1 protein with RAR.beta. selective retinoids and method for treatment of diseases and conditions with such retinoids

DEPR:
Retinoic acid receptors are a member of the steroid/thyroid receptor super family and they contain domains which are interchangeable within individual receptors. Thus, plasmids for chimeric retinoid receptors containing estrogen DNA binding domain and estrogen response element chloramphenicol acetyl-transferase enzyme are constructed and are grown in specific cultured bacteria. These plasmids respectively code for chimeric RAR.sub..alpha., RAR.sub..beta., RAR.sub..GAMMA., and if desired for testing RXR.sub..alpha., receptor proteins, and for the chloramphenicol acetyl A transferase (CAT) enzyme protein. The bacteria with these plasmids are obtainable in accordance with the procedure set forth in the article titled "Nuclear Retinoic Acid Receptors: Cloning, Analysis, and Function", M. Pfahl et al., Methods in Enzymology 189, p256-270 (1990) which is incorporated herein by reference. The detailed procedure how to isolate the DNA plasmids from the respective bacteria is also set forth below in detail, in the form of specific instructions under the title "Supercoiled Plasmid Isolation".

DEPR:
As it will be well understood by those skilled in the art, as a result of transfection with the respective DNA plasmid coding for RAR.sub..alpha., or RAR.sub..beta. etc. chimeric receptors and as a result of transfection with the ERA-CAT (which codes for the CAT enzyme as described above), the aforementioned plasmids are incorporated into the HeLa cells cultured in the assay. The retinoid receptor plasmids undergo transcription (into m-RNA) and subsequent translation into the corresponding chimeric receptor protein. Therefore, the Hela cell cultures obtained in this manner produce the respective RAR.sub..alpha., RAR.sub..beta., RAR.sub..GAMMA., or RXR.sub..alpha. chimeric receptor protein. As a result of transfection with the ERA-CAT, the cell cultures of this assay also contain the genetic information for manufacturing the CAT enzyme. However, as is noted above, the latter genetic information is not transcribed, and the CAT enzyme is not produced by the respective cell cultures of this assay, unless an appropriate agonist compound binds to and activates the respective RAR.sub..alpha., RAR.sub..beta., RAR.sub..GAMMA., or RXR.sub..alpha. chimeric receptor protein in the cell and this activated agonist-receptor complex binds to the estrogen response element of the ERE-CAT construct.

ORPL:
Andrea Fanjul, et al., "A New Class of Retinoids with Selective Inhibition of AP-1 Inhibits Proliferation", Nature, (1994) 372/3:107-111.

Document Number 1

Entry 1 of 4 File: USPT Mar 3, 1998

US-PAT-NO: 5723291
DOCUMENT-IDENTIFIER: US 5723291 A

TITLE: Methods for screening compounds for estrogenic activity

DATE-ISSUED: March 3, 1998

INVENTOR-INFORMATION:

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APPL-NO: 8/ 410807
DATE FILED: March 27, 1995

PARENT-CASE:
This is a continuation in part of U.S. Ser. No. 08/115,161 filed Sep. 1, 1993, now abandoned, which is incorporated herein by reference.

INT-CL: [6] G01N 33/68, G01N 33/74, C12Q 1/02, C12N 15/00

US-CL-ISSUED: 435/6; 435/7.1, 435/7.2, 435/7.21, 435/7.23, 435/69.1, 435/172.3, 435/29, 436/501, 536/24.1
US-CL-CURRENT: 435/6; 435/29, 435/69.1, 435/7.1, 435/7.2, 435/7.21, 435/7.23, 436/501 , 536/24.1

FIELD-OF-SEARCH: 435/6, 435/7.1, 435/7.2, 435/7.21, 435/7.23, 435/69.1, 436/501, 536/24.1

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PAT-NO	ISSUE-DATE	PATENTEE-NAME	US-CL
5071773	December 1991	Evans	436/501

FOREIGN PATENT DOCUMENTS

FOREIGN-PAT-NO	PUBN-DATE	COUNTRY
A 0 629697	December 1994	EP
WO A 9506754	March 1995	WO

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ART-UNIT: 181

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ABSTRACT:

The present invention provides novel assay methods for identifying compounds that may have both estrogen agonist and antagonist properties. In particular, the assay use cells comprising promoters having an AP1 site linked to a reporter gene. Compounds capable of inducing or blocking expression of the reporter gene can thus be identified. The compounds may be further tested for the ability to modulate the standard estrogen response, as well.

27 Claims, 23 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 12

BRIEF SUMMARY:

BACKGROUND OF THE INVENTION

Many breast tumors require estrogens for tumor growth. Thus, treatment with antiestrogen compounds can slow or prevent tumor spread. Many antiestrogens, however, show both estrogen antagonistic and agonistic activity. The nonsteroidal antiestrogen tamoxifen, for example, which is established as the treatment of choice for the endocrine therapy of advanced breast cancer, shows both agonistic and antagonistic activity. Sutherland, S. & Jackson, M. Cancer Treat. Revs. 15:183-194 (1987).

The agonistic activity of tamoxifen and other antiestrogens may have profound effects upon patients. For example, agonistic activity may have beneficial effects, such as preventing osteoporosis and reducing serum cholesterol. Love, et al. New Eng. J. Med. 326:852-856 (1992). Love, et al. J. Natl. Cancer Inst. 82:1327-1332 (1990). Conversely, agonistic activity may also be harmful. Tamoxifen for example sometimes increases endometrial tumor incidence Iino, et al. Cancer Treat. & Res. 53: 228-237 (1991) or switches from inhibition to stimulation of estrogen dependent growth in breast tumor progression. Parker, M. G. (ed) Cancer Surveys 14: Growth Regulation by Nuclear Hormone Receptors. Cold Spring Harbor Laboratory Press (1992).

It is desirable to identify pure antiestrogens as they are anticipated to provide more rapid, complete or longer-lasting tumor responses. Wakeling, A. E. Breast Cancer Res. & Treat. 25:1-9 (1993). For example, ICI 164,384 (hereinafter referred to as "ICI"), thought to be a pure antiestrogen, blocked MCF-7 cell invasion activity of a re-constituted basement membrane while estradiol and 4'-hydroxytamoxifen stimulated this activity suggesting that early treatment of breast cancer with a pure antiestrogen might be particularly beneficial in limiting tumor spread. Braacke, et al., Br. J. Cancer 63:867-872 (1991).

Conversely, while pure antiestrogens appear preferable for cancer treatments, mixed agonist-antagonist compounds may be preferable for preventative treatment. Such compounds should combine sufficient antagonist activity on estrogen stimulated breast tumor growth while maintaining simultaneous agonist activity on bone density and serum lipid levels.

In addition, a number of non-steroidal natural and synthetic compounds found in the environment have been shown to possess estrogenic activity. For instance, plant flavonoids including genistein and coumestrol and synthetic compounds such as phenolphthalein, alkylphenols, and dihydroxystilbenes, have been shown to be agonists of the estrogen receptor (Miksicek, Mol. Pharmacol. 44:37-43 (1993); Nieto et al., Biochem. Int. 21:305-311 (1990); White et al., Endocrinology 135:175-182 (1994); Makela et al., Environ. Health Perspect. 102:572-578 (1994); Krishnan et al. Endocrinology 132:2279-2286 (1993)).

Environmental estrogens, or xenoestrogens, are suspected of playing a role in the causation of a number of diseases such as breast and other cancers. In addition, such compounds may be implicated in human infertility and problems in wildlife reproduction. In the case of breast and other cancers, established risk factors (e.g., genetic factors) do not always account for the high levels of of these diseases. Evidence suggests that lifetime exposure to various xenoestrogens may be important in the induction of breast cancer. To the extent such xenoestrogens are important in diseases such as breast cancer, reduction in exposure to these compounds should be critical to reducing cancer risks (Davis et al. Environmental Health Perspectives 101:372-377 (1993)).

Currently, antiestrogen compounds or xenoestrogenic compounds are screened with animal models such as the rat uterine test. These tests are cumbersome, slow,

expensive and of uncertain application to humans because of differences between the human and rodent estrogen receptors.

The prior art fails to provide methods for quickly and easily testing potential antiestrogen compounds for agnostic as well as antagonistic properties mediated through pathways other than the classical estrogen response pathway, that may affect, adversely or beneficially, their use in various therapeutic applications. In addition, the ability to quickly and inexpensively screen environmental compounds for estrogenic activity is particularly important for assessing health consequences of new and existing chemicals. This invention addresses these and other problems in the art.

SUMMARY OF THE INVENTION

The present invention provides methods for screening test compounds, for example environmental compounds, for the ability to activate or inhibit transcription through an indirect estrogen response or classical estrogen response. The indirect estrogen response is mediated by promoters comprising an AP1 site and the classical estrogen response is mediated by promoters comprising a classical estrogen response element. Preferred AP1 sites can be isolated from metalloprotease genes. Preferred classical estrogen response elements can be isolated from the Xenopus vitellogenin A2 gene.

The methods typically use cells comprising an estrogen receptor and a promoter comprising an AP1 site which regulates expression of a reporter gene. The cells are then contacted with the test compound and the expression of the reporter gene is detected. The methods are conveniently used for testing compounds known to be antiestrogens for the ability to activate transcription through the AP1 site.

In other embodiments the assays are used to test environmental compounds for estrogenic activity. Environmental estrogens will typically be non-steroidal compounds, which are effective agonists of the estrogen receptor. Such compounds may be tested for their ability to activate transcription through the classical estrogen response element or through the AP1 site. For this purpose, cells which express mutant estrogen receptors are conveniently used. Cells expressing mutant estrogen receptors with lower activity are useful in decreasing background transcription. A preferred cell is the ERC1 cell line.

Cells derived from a source other than breast tissue are generally preferred for measuring activation mediated by the AP1 site. For example, uterine cells such as Ishikawa cells can be used. The reporter genes used to detect an estrogen response include genes encoding beta-galactosidase and bacterial chloramphenicol acetyl transferase. The promoters used may be those which naturally comprise AP1 or estrogen receptor elements or the promoters may be genetically engineered to comprise those elements.

In some embodiments a single cell may comprise two promoters, each with either the AP1 or the classical estrogen response element. In these embodiments, two different reporter genes are operably linked to the two promoters. In these assays the ability of test compound to induce or inhibit both the indirect and classical pathways can be determined.

When the methods are used to identify estrogen antagonists, the test compounds are contacted with the cells and a compound known to mediate an indirect estrogen response. The ability to inhibit the response is determined by detecting the expression of the reporter gene. Compounds known to mediate an indirect estrogen response include tamoxifen and estrogen at half maximal concentrations. The compounds can also be tested for the ability to induce or block the classical estrogen pathway, as well .

DRAWING DESCRIPTION:

BRIEF DESCRIPTION OF THE DRAWINGS

FIGS. 1A and 1B show estrogen stimulation mediated by an AP1 site in ERC1 cells (FIG. 1A) and F9 cells (FIG. 1B). Typical results of CAT assays, normalized for transfection efficiency, following a single transfection are shown opposite. Each point is the mean value of triplicate assays, with standard errors. CAT activities from cells maintained in the absence of hormone are shown as white bars, those in the presence of a saturating concentration (100 nM) of estradiol as black bars.

FIGS. 2A-2C show antiestrogen stimulation of expression in an intact AP-1 Site,

but not at classical EREs. FIG. 2A shows CAT assays of HeLa cells transfected with the indicated reporter genes and 3 .mu.g of human ER expression vector. Representations, at left, show the human collagenase promoter (shaded) and the consensus AP-1 site. CAT activities were from cells maintained in the absence of hormone or saturating concentrations of ICI 164,384 (ICI, 1 .mu.M), tamoxifen (5 .mu.M) or estradiol (100 nM). CAT activity is normalized to a transfection control with the actin promoter driving expression of .beta.HCG. Single representative experiments are shown, error bars represent standard deviation of triplicate hormone treatments. FIG. 2B shows CAT assays of HeLa cells transfected with reporter genes consisting of sequences overlapping the collagenase AP-1 site (-73 to -52) upstream of the herpes simplex virus TK promoter (from -109 to +45 relative to the start site of transcription) or the native TK promoter alone. FIG. 2C shows CAT assays of HeLa cells transfected with reporter genes containing classical EREs.

FIGS. 3A and 3B show that antiestrogen agonism at AP-1 sites requires ER. FIG. 3A shows dose dependence of estrogen and antiestrogen induction of coll73-LUC in HeLa cells relative to input ER expression vector, normalized to constant input DNA with blank expression vector SG5. The luciferase assays were normalized to actin-HCG and were expressed relative to values that were obtained with the collagenase promoter in the absence of expression vector and hormone. A single representative experiment with triplicate points is shown. Error bars represent standard deviations. FIG. 3B shows concentration dependence of estrogen and antiestrogen induction of coll73-LUC in HeLa cells. HeLa cells were transfected with 5 .mu.g of ER expression vector and the collagenase promoter active upon a luciferase reporter gene. The cells were exposed to a range of concentrations of ligand. Error bars represent standard deviation of triplicate points.

FIG. 4A and 4B show that tamoxifen is an agonist in endometrial Cells but not in breast cells. FIG. 4A shows the response of the transfected coll73-CAT reporter in Ishikawa cells treated with estrogen or antiestrogen. Left panel, coll73-CAT response with endogenous ER contrasted with the response of an ERE regulated reporter, ERE-coll60CAT. Right panel, coll73-LUC response with 3 .mu.g co-transfected expression vector for human ER. Averages of three individual experiments are shown. FIG. 4B shows activity of the collagenase promoter in breast cell lines with endogenous ER. Left panel shows response of the collagenase promoter driving CAT in MCF7 cells (average of four experiments). Right panel shows activity of the coil promoter driving luciferase expression in ZR75 cells, either without or with 300 ng of human ER expression vector. A single representative experiment with triplicate hormone treatments is shown.

FIGS. 5A-C show that hormone response at the AP-1 site requires AP-1 proteins. FIG. 5A shows potentiation of hormone responses in HeLa cells by Jun and Fos. Relative luciferase activities, normalized to HCG production, and calculated relative to collagenase expression in the absence of ER and hormones are presented. The errors represent standard deviations of three separate experiments. FIG. 5B shows effects of ER with and without transfected Jun and Fos on hormone induction of the collagenase promoter in F9 cells. Averages of five or six individual transfections are shown. FIG. 5C shows response of the collagenase promoter in F9 cells to increasing amounts of Jun, Fos, or their combination, in the absence of ER.

FIGS. 6A-C show that the DNA binding domain of ER is required for tamoxifen induction at an AP-1 site, but not required for estrogen induction. Reporters regulated by an AP-1 site (left panels), or an ERE (right panels) were introduced into Hela (FIG. 6A), CHO (FIG. 6B), or MDA453 cells (FIG. 6C), with 5 .mu.g, 100 ng and 1 .mu.g respectively of each expression vector for the ER derivative whose structure is indicated. The DNA binding domain is indicated with the striped box, the ligand binding domain (AF2) and the amino terminal (AFI) activation functions are marked. Results are presented for coll73-LUC in HeLa and MDA453 cells and coll73-CAT in CHO cells. CAT and luciferase activities are calculated relative to those obtained with coll73-LUC or coll73-CAT with SG5 blank expression vector in the absence of hormones.

FIGS. 7A and 7B show that fusing an exogenous transactivation function to the ER increases activation at AP-1 sites. A luciferase reporter regulated by an AP-1 site (left panels) and a CAT reporter regulated by an ERE (right panels) were introduced into Hela (FIG. 7A), CHO (FIG. 7B) with expression vector for the ER derivative whose structure is indicated. CAT or Luciferase activities, normalized to HCG production are shown. Activator plasmids, are shown schematically at the left of the figure. The VP16 transactivation domain is represented as an oval. The GALA DNA binding domain is marked.

FIGS. 8A and 8B show that fusing an exogenous transactivation function to an ER

derivative without the ligand binding domain potentiates gene expression mediated by an ERE but not by an AP-1 site. A luciferase reporter regulated by an AP-1 site (left panels) and a CAT reporter regulated by an ERE (right panels) were introduced into HeLa cells with expression vector for ER derivatives. CAT or Luciferase activities, normalized to HCG production, are shown.

FIG. 9 shows a library of hydroxystilbene derivatives tested in the assays of the invention.

FIG. 10 shows estrogenic activity of series 4 compounds illustrated in FIG. 9 as well as inhibition of activity by treatment with ICI.

FIG. 11 shows dose response curves for series 4 compounds.

FIG. 12 shows results of ER binding/competition assays for series 4 compounds.

DETAILED DESCRIPTION:

DESCRIPTION OF THE PREFERRED EMBODIMENT

The present invention provides an efficient way to screen large numbers of test compounds for those which have desirable properties for either the treatment or the prevention of various cancers (e.g. breast cancer, ovarian cancer, endometrial cancer) and other diseases (e.g. endometriosis) mediated by estrogen. The invention thus provides methods of screening for novel types of antiestrogen compounds that block the indirect estrogen response and/or block estrogen action at classical estrogen response elements. As used herein an antiestrogen is a compound that substantially inhibits estrogen activity as measured in a standard assay for estrogenic activity, for example, cellular assays as described in Webb et al. Mol. Endocrinol., 6:157-167 (1993).

The invention also allows for screening of test compounds for estrogenic activity. The assays are particularly useful for screening environmental compounds suspected of having estrogenic activity, referred to here as xenoestrogens. Xenoestrogens are defined here to include any compound having estrogenic activity in the assays described herein, which is derived from a source outside the human body. Environmental compounds as used herein can be derived from a wide variety of sources including plants, soil, water, foods. They also include synthetic compounds such as chlorinated organics, polycyclic aromatic hydrocarbons, herbicides, pesticides, pharmaceuticals and the like.

In animals and in man the balance between stimulatory and inhibitory activities of antiestrogens such as tamoxifen varies widely depending on the organ, cell or specific protein measured as an indicator of estrogenic activity. This variety of effects is difficult to reconcile with the model of antagonism of estrogen receptor (ER) activity at classical estrogen receptor elements (EREs) as described in Beato, M. Cell, 56: 335-344 (1989) and Klein-Hitpass, et al., Nucleic Acids Res., 16:647-663 (1988).

The present invention relies, in part, on the discovery that ERs may activate transcription by interaction with another response element, the AP i binding site, instead of binding to EREs. This AP1 mediated pathway, referred to here as the indirect estrogen response, may account for much of the agonistic properties of tamoxifen and other putative antiestrogens. A general description of the AP1 site is found in Angel & Kann, Biochem. Biophys. Acta., 1072:129-157 (1991) and Angel, et al., Cell, 49: 729-739 (1987).

In the methods of the invention, both the classical estrogen response elements and the indirect estrogen response may be used to provide a screening system that detects both estrogen antagonistic and agonistic activity. When testing an environmental compound for estrogenic activity, the methods typically comprise cultured cells that produce high levels of the human estrogen receptor. Such cells include MCF-7 cells (ATCC No. HTB 22), MDA453 cells (ATCC No. HTB 131), ZR-75-1 cells (ATCC No. CRL 1500) or ERC1 cells described in Kushner et al., Mol. Endocrinol., 4:1465-1473 (1990). ERC2 and ERC3 cells as described by Webb, et al. Mol. Endocrinol., 6:157-167 (1993).

Cells expressing mutant estrogen receptors with decreased sensitivity for estrogenic compounds are preferred for testing environmental compounds. Cells expressing the wild type receptor (e.g., MCF7 cells) have high background levels of transcription even in the absence of hormone. Transcription induced by weakly active environmental compounds may be masked in these cells. Thus, preferred cells for this purpose include cells which over-express mutant estrogen

receptors, such as the ERC cells noted above.

When testing an antiestrogen compound's ability to activate transcription through the AP1 mediated pathway, the source of the cells used in the assay can influence the results. In particular, evidence provided below indicates that agonistic activity of an antiestrogen (e.g., tamoxifen) is usually strong in cells in which its agonistic activity at an ERE is weak, and weak in cells in which its agonistic activity at an ERE is strong. As shown below, antiestrogens show little or no agonistic activity through the AP1 pathway in breast cancer cell lines. Thus, when testing antiestrogens for agonistic activity in the AP1 pathway, cells other than breast cancer cell lines are usually used. For instance, cells of uterine origin such as cervical cells (e.g., HeLa cells) or endometrial cells (e.g., Ishikawa cells) can be used. The invention is not limited to practice in mammalian cells and may be practiced, for example in yeast and insect cells, transfected with the appropriate genes and recombinant constructs.

The cells may be modified to provide truncated or chimeric estrogen receptors as described in Berry, et al., E. M. B. O. J., 9:2811-2818 (1990). These modifications may result in increased estrogen affinity and increased sensitivity of the assay.

In addition, these cells are transfected with reporter genes in which a response element (either the AP1 site or ERE) regulates expression of a reporter gene. Typically, two different reporter genes are used. One gene reports transcription induced by the classical estrogen response system, while the other gene reports transcription induced by the indirect estrogen response. The two reporter genes and response elements are typically placed in separate cells, but the methods can also be used with both constructs in the same cell.

The reporter gene for the classical estrogen response system contains an estrogen response element (ERE) upstream of the target promoter and capable of regulating that promoter. In a preferred embodiment the ERE may be the consensus estrogen response element AGGTCACAGTGACCT (SEQ ID NO: 1) from the Xenopus vitellogenin A2 gene.

The particular ERE used in the cells is not a critical aspect of the invention and the present invention is not limited to the use of this ERE. Other EREs known to one of skill in the art can also be used. For instance, other sources of naturally occurring EREs include the B2 gene, the chicken ovalbumin gene, and the PS2 gene. Alternatively, non-naturally occurring EREs may be inserted into particular promoters. The consensus ERE from the Xenopus vitellogenin A2 gene is widely used for this purpose, but other EREs may be used as well.

The reporter gene for the indirect estrogen response pathway contains an AP1 site upstream of the target promoter and capable of regulating that promoter. The AP1 site is a sites that are bound by AP1 (the Jun and Fos proteins) or other members of that protein family. In a preferred embodiment, the consensus AP1 site is TGA(C/G)TCA.

One of skill would recognize that the particular AP1 site used is not a critical aspect of the invention. Any sequence capable of being bound by AP1 or members of that family and regulating a promoter is suitable. This would include promoters which encompass a naturally occurring AP1 site. Typical promoters include, but are not restricted to metalloprotease genes such as stromelysin, gelatinase, matrilysin, and the human collagenase gene.

Alternatively promoters may be constructed which contain a non-naturally occurring AP1 or related binding site. This facilitates the creation of reporter gene systems that are not typically found under the control of AP1. In addition, promoters may be constructed which contain multiple copies of the AP1 site thereby increasing the sensitivity or possibly modulating the response the reporter gene system.

The present invention is not limited to a particular reporter gene. Any gene that expresses an easily assayable product will provide a suitable indicator for the present assay. Suitable reporter genes are well known to those of skill in the art. They include, for example, bacterial chloramphenicol acetyl transferase (CAT), beta-galactosidase, or luciferase.

One of skill will recognize that various recombinant constructs comprising the AP1 site can be used in combination with any cell or line which expresses a estrogen receptor.

To screen a number of compounds for antiestrogen action, cells with high level

expression of human estrogen receptors and harboring either or both response elements and reporter genes are exposed to doses of estrogen which give half maximal induction or less. In each ease this will result in induction of several to hundreds of fold depending on the levels of estrogen receptor and the particular details of the reporter construction. This will be reflected in increases of the reporter gene product, such as the CAT gene product which may be quantitated by enzymatic assay. The cells can be exposed to estrogens either growing in separate wells of a multi-well culture dish or for coloromectic assay in a semi-solid nutrient matrix. The antiestrogenic compounds to be tested are added to the culture dish wells or to small wells made in the semi-solid matrix and the effect on the estrogen induction is assayed. An antiestrogen compound will reduce or abolish the estrogen induced increase in reporter gene activity. A hypothetical pure antiestrogen will block estrogen action with both types of reporter genes and will have no ability to induce the reporter genes in the absence of estrogen. A mixed estrogen antagonist-agonist, will show some ability to induce the reporter genes, especially the reporter genes linked to AP1 site.

In other embodiments, environmental compounds suspected of having estrogenic activity are contacted with cells with high level expression of human estrogen receptors and harboring either or both response elements and reporter genes as described above. Those compounds with estrogenic activity will result in induction of several to hundreds of fold depending on the levels of estrogen receptor and the particular details of the reporter construction. Quantification of the activity by enzymatic assay can be carried as described above.

An assay for detection of xenoestrogens can be conveniently provided in kit format. Such a kit includes growth media, estrogen standards, and cells comprising the appropriate recombinant constructs. The kits may also include reagents suitable for detecting the product of the reporter .gene, and the like.

Antiestrogens which block the indirect pathway can be used to supplement tamoxifen or other antiestrogens in the treatment or prevention of breast cancer and other diseases mediated by estrogen. These compounds function to eliminate estrogenic agonistic activity of antiestrogens. Second they may have uses by themselves. In particular, it may be advantageous to block some estrogen mediated pathological effects at indirect estrogen response elements while leaving the direct pathway active. Compounds that block the indirect pathway are useful as components of combined oral contraceptives (COC) containing estrogens and progestins. A triple COC, containing estrogens, progestins, and a blocking compound would allow estrogen, either in the formulation or endogenous to act at the classical response elements, but would block action at the indirect response elements. Thus, a triple COC functions as current COCs to prevent pregnancy, but may also provide protective effects against breast cancer.

Typically, the reporter gene linked to the AP1 site is activated, not with estrogen, but with an excess (10 times the Kd) of tamoxifen, 4-hydroxy tamoxifen, or other antiestrogen. A library of compounds is searched for candidates that block or reduce reporter gene activation by the antiestrogen. The candidates are then tested with cells containing the reporter gene for the classical pathway to confirm that they do not interfere with estrogen activation at an ERE.

Antiestrogen or estrogen compounds identified in the assays of the invention can be used in standard pharmaceutical compositions for the treatment of cancer, as components of oral contraceptives, or any other application in which the modulation of estrogen activity is desired. The pharmaceutical compositions can be prepared and administered using methods well known in the art. The pharmaceutical compositions are generally intended for parenteral, topical, oral or local administration for prophylactic and/or therapeutic treatment. The pharmaceutical compositions can be administered in a variety of unit dosage forms depending upon the method of administration. For example, unit dosage forms suitable for oral administration include powder, tablets, pills, and capsules.

Suitable pharmaceutical formulations for use in the present invention are found in Remington's Pharmaceutical Sciences, Mack Publishing Company, Philadelphia, Pa., 17th ed. (1985). A variety of pharmaceutical compositions comprising compounds of the present invention and pharmaceutically effective carriers can be prepared.

The following examples are offered by way of illustration, not by way of imitation.

EXAMPLE 1

MATERIALS AND METHODS

Plasmid Construction

All reporter genes described below have been modified by digestion with Eco0109 and Ndel to remove an AP-1 site in the backbone of pUC. Thus, Coll73 and Coll60 are formerly .DELTA.Coll73 and .DELTA.Coll60 (Lopez et al. Mol. Cell. Biol. 13:3042-930 (1993)). Coll73-LUC was constructed by cloning a BamHI/PvuII fragment, that spanned the luciferase transcription unit, from pMG3 into coll73, which had been digested with BamHI and Sinai to remove the CAT transcription unit. EREcoll60 and EREcoll73 was prepared by ligation of a consensus ERE (AGGTCACAGTGACCT SEQ ID NO: 1), into the HindIII site upstream of coll60 and coll73, respectively. All other reporter genes have been previously described (Webb et al. Mol. Endocrinol. 6:157-16725 (1992); and Lopez et al., supra).

Expression vectors for ER and ER mutants (Kumar et al. Cell 51:941-51 (1987)), VP16-ER (Elliston et al. J Biol Chem 265:11517-21 (1990)), c-jun (Turner et al. Science 243:1689-94 (1989)) and c-fos (Sassone et al. Cell 54:553-60 (1988)) have been described. For this study, all ER cDNAs were cloned into the EcoRI site of the SG5 expression vector (Green et al. Nucleic Acids Res 16:369 (1988)).

The VP16ER cDNA was also cloned into SG5, to form the vector VER, in two steps. The expression vector Vp16ER1-422 (Elliston et al., supra) was digested with SstI, repaired with T4 polymerase, and ligated to ECORI linkers. The resulting EcoRI fragment was sub-cloned at the equivalent site of SG5 to generate the vector VER1-422. This was digested to completion with HindIII and BgIII and the equivalent (HindIII/BamHI) fragment from HEO was replaced at this location. VER.DELTA.DBD was constructed by substituting a NotI/BgIII fragment from HE11 (cloned in SG5) into VER digested with the same enzymes.

The GST wild type ER fusion gene (GST-HEGO) was constructed by ligation of the EcoRI fragment from pSG5-HEGO, spanning the ER cDNA, into pGEX5X-1, one of the vectors of the pGEX series (Pharmacia Biotech Inc., Piscataway, N.J., USA). GST-hELBD was constructed in two steps. An Xba fragment from HE19G was inserted into the equivalent position of XbaI digested SG5-HE14, which spans the ER LBD (Kumar et al.). Then, an EcoR1 fragment spanning this ER cDNA was cloned into pGEX-3X. To prepare GST-hEN185 an EcoRI/KpnI fragment spanning the ER amino terminus was obtained from the vector EGE, repaired and cloned into pGEX-5X-1 digested with SmaI and EcoRI.

Tissue Culture and Transfections

Cells were maintained and transfected as previously described in Webb et al., supra. Hormones were added two hours after plating in the following concentrations, estradiol 100 nM, ICI 164,384 1 .mu.M, tamoxifen 5 .mu.M, to ensure saturation of the response. F9 cells were seeded at 30% confluence upon 1.5 cm dishes and transfected overnight by calcium phosphate coprecipitation with 5 .mu.g reporter gene, 1 .mu.g actin .beta.-HCG, and 1 .mu.g of HEO, 300 ng each of c-jun and c-fos expression vectors. The cells were glycerol shocked and refed in growth medium containing hormone or ethanolic vehicle. In transient transfections, optimal mounts of HEO were employed and were as follows: HeLa (5 .mu.g); NIH3T3 (1 .mu.g); HepG2 (1 .mu.g); SHM (300 ng); SY5Y (300 ng); CEF (100 ng); CV-1 (3 .mu.g); MDA453 (3 .mu.g); CHO (100 ng) and F9 (1 .mu.g).

CAT assays were carried out as described in Webb et al., except that the cells were harvested one to two days after transfection instead of three. CAT activities were defined as the increase in cpm per hour at room temperature (corrected for background) per 100 .mu.l of cell extract, normalized to production of 100 standard units of .beta.HCG, from a co-transfected reporter gene, actin-HCG. Luciferase assays were performed as described by Brasier et al. Methods Enzymol 216:386-97 (1992)) on similar extracts that were used for CAT assays. Light units were defined as the luciferase activity per 100 .mu.l of cell extract per 100 standard units of .beta.HCG. Relative luciferase activities were calculated with respect to the results that were obtained in the absence of ER and hormone, which was set at 1 unit. For the data presented in Table I triplicate points were determined. Standard deviations were less than 20%.

GST Fusion Protein Binding Assay and in vitro Translation

Procedures were carried out as described by Lopez et al., supra. Briefly, fusions of GST to various domains of the human ER were prepared as follows. Bacteria expressing the fusion proteins were resuspended in buffer IPAB-80 (20 mM REPES, 80 mM KCl, 6 mM MgC!2, 10% Glycerol, 1 mM DTT, 1 mM ATP, 0.2 mM PMSF and protease inhibitors; pH 7.9), sonicated mildly, and the debris was pelleted at 12,000 rpm for 1 hr in an ss34 rotor. The supernatant was incubated for 2 hrs. with 500

.mu.L of glutathione sepharose 4B beads that were previously washed with 5 volumes of PBS 0.2% Triton X-100 and equilibrated with 5 volumes of IPAB 80. GST-fusion proteins beads were then washed with 5 volumes of PBS 0.05% Nonidet P-40 and resuspended in 1 ml of IPAB-80 for storage at 4 C until use. All the above procedures were done in a cold room at 4 .degree. C.

Assays of GST-ER fusions were carded out in 100 .mu.L volume that contained 40 .mu.L of bead suspension (equivalent to 10 .mu.L of compact beads volume) and 1 to 2 .mu.L of 35S in vitro translated c-jun or c-fos in IPAB-80 2.5% non fat milk and incubated for 1.5 hr at 4.degree. C. Beads were washed 5 to 6 times with IPAB-80 containing 0.05% Nonidet P-40. Input labelled proteins, proteins bound to GST, GST-hER and other ER fusion beads were then subjected to SDS polyacrylamide gel electrophoresis (PAGE) in 10% acrylamide and then to autoradiography.

RESULTS

Antiestrogens activate transcription through the AP1 site.

The human collagenase gene, like other matrix metalloproteases, responds to AP1. The promoter from this gene contains a consensus AP-1 site located between -60 and -73 base pairs from the start of transcription. Angel, et al., Mol. Cell. Biol., 7: 2256-2266 (1987). To test whether an AP1 site could confer estrogen response the collagenase promoter was fused to the bacterial CAT gene (.DELTA.coll73) and transfected into Chinese Hamster Ovary cells that over-express ER (ERC1) Kushner, et al. Mol. Endocrinol. 4:1465-1473 (1990).

Estradiol stimulated .DELTA.coll73 ten fold (FIG. 1A), whereas a similar reporter in which the AP1 site had been removed (.DELTA.coll60), gave reduced basal activity and no estrogen response. Substitution of a classical ERE (Klein-Hitpass, et al., Nucl. Acids Res. 16:647-663 (1988)) for the AP1 site (ERE.DELTA.coll60), restored estrogen response, but not the elevated basal activity.

In F9 cells, which lack endogenous AP1 activity (Chiu, et al. Cell, 54: 541-551 (1988)) .DELTA.coll73 failed to respond to estrogen in the presence of transiently transfected ER (FIG. 1B). Estrogen activation could be restored by cotransfecting expression vectors for AP1 proteins c-Jun and c-fos. Sassone-Corsi, P. et al., Cell, 54: 553-560 (1988). Turner & Tjian, Science 243:1689-1694 (1989). In parallel, ERE.DELTA.coll60 was estrogen responsive, even in the absence of AP1, and .DELTA.coll60 and remained unaffected by estrogen (data not shown). Estrogen induction of the collagenase promoter therefore required both the AP1 site and AP1 proteins.

To further examine the effects of antiestrogens on the AP-1 directed pathway, reporter genes derived from the human collagenase promoter were transfected into HeLa cells. Both estrogen and antiestrogens activated the collagenase promoter in the presence of transiently expressed human ER (coll517, FIG. 2A). In these cells tamoxifen was more potent an activator than estrogen. This pattern was retained with coll73, but was lost with coll60 or was inactivated by point mutations (coll517mAP1).

When the collagenase AP-1 site was placed upstream of the herpes virus tk promoter both tamoxifen and ICI were able to activate transcription, although this response was not as robust as with the native collagenase promoter (FIG. 1B). These results indicate that antiestrogens are agonists at the collagenase promoter and a heterologous promoter linked to AP-1. Thus, the AP-1 site is required for this activity.

The activity of antiestrogens in the AP-1 pathway was also compared with their activity in the classical pathway. Direct substitution of an ERE for the collagenase AP-1 site restored estrogen response to the core collagenase promoter, but not antiestrogen response or the basal activity associated with the AP-1 site. A promoter with both an ERE and an AP-1 site (ERE-coll73 FIG. 2C) gave a large estrogen response, but retained some response to antiestrogens. Another control reporter, in which the tk promoter was regulated by a classical ERE (ERE-tk FIG. 2C) was also activated by estrogen, but not by antiestrogens. Thus, a classical ERE cannot substitute for the AP- 1 site, indicating that the AP-1 site has a unique function in activation by antiestrogens.

To determine whether ER was required for antiestrogen agonism the response of the collagenase promoter, upstream of the luciferase gene (coll73-LUC), to transfection of increasing mounts of ER into HeLa cells was examined (FIG. 3A). Estrogen and antiestrogen responses were not seen in the absence of ER, and increased as a function of the mount of transfected ER expression vector.

Tamoxifen responses were more potent than estrogen responses at every level of receptor.

The effect of increasing doses of each ligand was also examined. FIG. 3B shows that the half maximal dose for ICI is about 10 times, and tamoxifen 100 times, that for estrogen. This is consistent with the known binding affinities of these compounds to the estrogen binding site on the receptor and suggests that they are stimulating transcription through that site. Similar half maximal doses were obtained for both estrogen and the weak tamoxifen responses that were seen at a classical ERE (dam not shown).

In summary, indirect estrogen response is widely active, and antiestrogens are agonists of this pathway. It is possible that any of the well described agonist effects of tamoxifen reflects indirect estrogen response. Antiestrogens would have estrogenic activity on critical AP1 regulated target genes, hence growth and differentiated response, in cells in which ER and AP1 proteins could interact. Changes in AP1 during tumor progression could be particularly significant, and should be considered in models of antiestrogen resistance in breast cancer. Parker, et al. Cancer Surveys, 14, Growth Regulation by Nuclear Hormone Receptors. Cold Spring Harbor Laboratory Press (1992).

Antiestrogens Are Agonists of the AP-1 Pathway in Many Cell Types

The data above show that antiestrogens are agonists at the AP-1 driven collagenase promoter, but not at classical EREs, in HeLa and other cells. To test whether this pattern was widespread, the effect of estrogen and antiestrogens on the expression of reporter genes driven by either the native collagenase promoter, or a similar promoter in which the AP-1 site was replaced by a classical ERE, was tested in a range of cell lines. In each case, the cells were transfected with different amounts of the human ER expression vector HEO to determine the optimal response.

Table I shows that both estrogen and antiestrogens activated the collagenase promoter in most cell types. This response occurred with cell lines representative of different tissue types including cervix, liver, myometrium, neuroblastoma, kidney and ovary. In most cases tamoxifen was as potent, or more potent, than estrogen. Only F9 cells, which have low levels of endogenous AP-1 activity, were not activated by any ligand.

In the same range of cell types both antiestrogens displayed little activity at classical EREs (data not shown). ICI consistently behaved as a pure antagonist of ER action at an ERE. In HeLa cells, and most other cases, tamoxifen inductions of ERE-coll60 activity remained at less than 3% of those obtained with estrogen. Significant (30% of estrogen) tamoxifen inductions at classical EREs were obtained in CEF cells and CV-1 cells and MDA453 cells. In these latter cells tamoxifen action at the AP-1 site was relatively weak (Table I). Thus, tamoxifen activity at an AP-1 site may be strong in cells at which its activity at an ERE is weak (HeLa), and weak at an AP-1 site in cells at which its activity at an ERE is strong (MDA453).

In conclusion, antiestrogen agonist effects occur at AP-1 sites in cells of diverse origin. These effects show little correlation with the activity of tamoxifen at classical EREs.

Antiestrogens are Agonists of the AP-1 Pathway In Endometrial Cell Lines, But Not in Breast Cells

The data in Table I show that, in most cell types, tamoxifen was at least as potent as estrogen in inducing the collagenase promoter. In one cell line, MDA453 breast cancer cells, the AP-1 driven collagenase promoter was activated efficiently by estrogen but not by tamoxifen. Similarly, in Chinese hamster ovary (CHO) cells, estrogen inductions routinely exceeded antiestrogen inductions. This suggests that tamoxifen action at the collagenase promoter might have a cell specific component.

To further explore this phenomenon, and to test whether similar hormone effects could be detected at physiological levels of ER, the expression of the collagenase promoter in cells that express endogenous ER was examined. Ishikawa cells, an endometrial cell line that is believed to represent a model of tamoxifen agonism on the uterus (described by Holinka, et al., J. Steroid Biochem., 25:781-786 (1986)), and two breast cancer cell lines, MCF-7 and ZR-75-1, both of which are known to respond to estrogen but not tamoxifen, were used.

In Ishikawa cells (FIG. 4A) the collagenase promoter was activated by estrogen and tamoxifen, but the ICI compound was usually inactive in the presence of endogenous receptor. This parallels the reported potency of tamoxifen and ICI on cell growth and induction of progesterone receptors in these cells. When receptor levels were raised by transfection, tamoxifen and estrogen inductions became larger and ICI inductions became detectable. In contrast, neither ICI nor tamoxifen activated expression of ERE-coll60, whereas estrogen induced expression of this reporter gene tenfold. Tamoxifen also failed to activate several other genes that contain simple classical EREs in Ishikawa cells (data not shown).

In MCF-7 cells, estrogen, but not tamoxifen, activated the collagenase promoter (FIG. 4B). The same pattern occurred in ZR-75-1, and could be seen more clearly when extra receptors were supplied by transfection. Again, this resembles the results that were obtained in MDA453 cells (Table I), and parallels the reported absence of tamoxifen effects on cell proliferation and gene expression in breast cancer cell lines.

In conclusion, tamoxifen activates the collagenase promoter in cells with physiological levels of ER, and the response shows tissue restrictions. Tamoxifen activity occurs in endometrial cells, but not in breast cells, and thus parallels the known tissue specificity of tamoxifen agonism.

TABLE I Coll73 Luciferase Activity (*1)

Cell Line	Origin	Hormone	ICI	Tam	E2
HELA	CERVIX	1.0	3.7	7.4	3.4
NIH 3T3	FIBROBLAST	1.0	3.0	3.1	3.3
HEP G2	LIVER	1.0	5.2	6.5	4.3
SHM	MYOMETRIUM	1.0	1.9	2.2	2.0
SY5Y	NEUROBLASTOMA	1.0	2.3	2.1	2.1
CEF	FIBROBLAST	1.0	3.2	2.2	2.5
CV-1	KIDNEY	1.0	3.1	5.3	2.2
MDA453	BREAST	1.0	1.1	2.	8.4
CHO	OVARY	1.0	1.8	2.2	3.3
F9 (*2)	TERATOCARCINOMA	3.2	2.4	1.1	1.4

(*1) Activities were determined in triplicate transfections. Activities were normalized to an actinHCG internal control and expressed relative to values obtained from the collagenase promoter in cells that were not transfected with ER or treated with hormone (see Materials and Methods). Standard deviations (not shown) were less than 20%. (*2) Unliganded ER increased the basal activity of the collagenase promoter.

AP-1 Proteins Are Required for ER Action at the Collagenase Promoter

To test whether AP-1 proteins, as well as their cognate binding site, were required for the AP-1 pathway, we examined whether Jun and Fos overexpression affected the hormone response of the collagenase promoter. The Examples above establish that antiestrogens and estrogens activate the collagenase promoter in HeLa cells in the presence of ER (see, e.g., Table I). FIG. 5A shows that these inductions are markedly increased by the presence of transfected AP-1, especially in the presence of Jun or Jun/Fos. This suggests that Jun homodimers or Jun/Fos heterodimers occupying the AP-1 site contribute to the ability of ER to activate description in the AP-1 directed pathway.

To confirm that AP-1 proteins were absolutely required for the AP-1 directed ER pathway, we turned to F9 cells, which have only low levels of endogenous AP-1 activity. Transfection of an expression vector for estrogen receptor into these cells did not support hormone activation of the collagenase promoter (Table D, whereas it gave strong estrogen activation at an ERE (not shown). Co-transfection of ER with Jun/Fos restored induction by both estrogen and antiestrogens in F9 cells, albeit at lower levels than that seen in HeLa cells. In addition there was some activation by unliganded ER. Thus, the inability of F9 cells to allow a hormone response at the collagenase promoter can be overcome with AP-1 supplied by transfection. We conclude that hormone effects at the AP-1 site require AP-1 protein. However, the dramatic difference between the hormone response of HeLa and F9 cells when both are supplied with Jun and Fos indicates that other cell specific factors, in addition to AP-1 abundance, regulate the strength of the AP-1 directed ER pathway.

It is unlikely that ER dependent activation at AP-1 sites is due to changes in the mount of AP-1. In these studies we determined the mounts of AP-1 required for optimal collagenase promoter activity in F9 cells. FIG. 5C shows that Jun, Fos, and a combination of both, increased basal activity of the collagenase promoter (in the absence of ER) which reached a maximum with 300 ng of expression vector. These mounts were employed in the co-transfections with ER (FIG. 5B). Thus, ER activation at AP-1 sites appears to increase the transcriptional efficiency of Jun and Fos even when they are provided at optimal mounts.

ER Binds Jun But Not Fos in vitro

To test whether ER effects upon AP-1 might reflect direct biochemical interaction

between the ER and AP-1 proteins, we examined whether they specifically interact in solution. An estrogen receptor protein fused to glutathione S-transferase (ER-GST), and attached to agarose beads, pelleted in vitro translated Jun from solution, whereas a control GST protein pelleted only background amounts of Jun. Similar binding occurred with the ER amino terminal domain, but not with the LBD. Neither the intact ER nor its isolated domains bound Fos. These results indicate that Jun, but not Fos, binds ER in vitro, and that a major target of Jun is the ER amino terminus.

Tamoxifen Activation at AP-1 Requires the ER DBD, Whereas Estrogen Activation Is DBD Independent In Some Cell Types.

We next examined which domains of the ER mediate hormone action. We introduced truncated derivatives of the ER into three different cell types. We chose the HeLa, CHO and MDA453 lines as recipients because the ER driven AP-1 pathway showed different properties in each cell. In HeLa cells tamoxifen response predominated, in MDA453 cells estrogen response predominated, and CHO cells gave an intermediate phenotype (Table I). We examined the ability of each truncated ER to activate a reporter gene driven by the collagenase promoter with its AP-1 site (FIG. 6, left side) or a reporter gene driven by control promoter with an ERE (FIG. 6, right side). Previous work has established that each of these variant ERs is expressed at comparable levels from these vectors.

Deletion of the DNA binding domain (DBD) completely eliminated estrogen activation at an ERE in all three cell types (HE11, FIG. 6). Deletion of the DNA binding domain also eliminated tamoxifen activation at AP-1 sites, be it the substantial tamoxifen activation in HeLa and CHO cells, or the marginal mount in MDA cells. In contrast, removal of the DBD did not abolish estrogen activation at the AP-1 site in any of the cell lines. Indeed, estrogen activation at the AP-1 site in CHO cells was equally strong with or without the ER DBD. This is consistent with previous observations that estrogen response at AP-1 sites shows independence of DNA binding in CEF. Thus, the requirement for the ER DBD varies according to the ligand, it is required for tamoxifen induction but not estrogen induction. We suggest below (Discussion) that the differential requirements for the ER DBD may indicate the existence of more than one pathway of ER action at AP-1 sites.

The ER amino terminus also played an important role in tamoxifen and estrogen activation at the AP-1 site. Although deleting of the amino terminus (HE19) did not eliminate activity upon the ERE regulated reporter in all three cell types, this deletion abolished the strong tamoxifen-activation at the AP-1 site in HeLa cells and the weaker tamoxifen activation in CHO and MDA453 cells. Deletion of the amino terminus also markedly reduced estrogen activation at the AP-1 site in all three cell types.

A deletion of the ligand binding domain (HE15), leaving the amino terminus and DBD intact, gave a constitutively active receptor that was able to weakly activate at an ERE in all three cell lines. This receptor, however, showed highly potent activity at the AP-1 site in HeLa cells, which correlated with the levels of activity obtained with the tamoxifen liganded native ER. In contrast, HE15 was inactive in MDA453 cells and weak in CHO cells. Thus, the requirement of the ER amino terminus for AP-1 activation also shows cell type specificity, in a manner that correlates with the cells ability to support a tamoxifen response at the collagenase promoter. This again suggests that activation through AP-1 may occur through more than one mechanism.

ER Can Target an Exogenous Transactivation Domain to the Collagenase Promoter, Independently of the ER DBD

One possible mechanism for ER activation at AP-1 sites is that the receptor might directly bind to the AP-1 complex at the promoter and from there influence transcription. A prediction of this model is that ER should be able to target heterologous transcriptional activation functions to an AP-1 regulated promoter.

In order to test this proposition, we examined the effects of linking the strong VP16 transcriptional activation domain to the amino terminus of the ER (V-ER). To monitor activity we used a luciferase reporter gene regulated by an AP-1 site and CAT reporter gene driven by an otherwise identical promoter with an ERE. The V-ER chimeric receptor gave markedly enhanced activation at an ERE in HeLa cells (FIG. 7A). It was activated both by estrogen and antiestrogens reflecting the ability of VP16 to override the need for AF-2 (see, Kumar et al. Cell 51:941-951 (1987)), and consistent with previous reports for this "super-receptor". In contrast, the super-receptor had little effect at the AP-1 site in HeLa. Tamoxifen activation with the full length ER was hardly increased, although estrogen activation was

modestly potentiated. We also tested a version of the super-receptor in which the ER DBD was deleted (VER.DELTA.DBD). This receptor, as expected, failed to activate at an ERE. It was, however, more potent than an equivalent ER (HE11) that lacked the VP16 activation function when tested at an AP-1 site.

To further explore this phenomenon we performed a series of similar experiments in CHO cells (FIG. 7B), in which estrogen response at the AP-1 site was completely independent of the ER DBD (FIG. 6B). Once again, the V-ER chimera superactivated gene expression that was driven by the ERE. In this case, however, V-ER also superactivated at the AP-1 site. Although the superreceptor that lacked the DBD (VER.DELTA.DBD) remained unable to activate transcription from an ERE, it was even more active than V-ER at the AP-1 site. A control fusion of the VP16 domain to the yeast GAL4 DNA binding domain did not increase collagenase promoter transcription. Thus, the superactivation by VP16 in CHO cells is dependent upon sequences in the ER protein. These observations indicate that super-receptors are super-activators at AP-1 sites in CHO cells in a DBD-independent manner. Similar results were also obtained with MDA453 cells (data not shown). The contrast between the properties of the super-ER in HeLa and CHO cells further suggests that there may be more than one pathway of activation at AP-1 sites.

VP16 Potentiates the Action of an ER Without an LBD at an ERE, But Not at an AP-1 Site.

The results described above suggest that addition of the VP16 activation function to native ER was unable to strongly potentiate tamoxifen action at an AP-1 site in HeLa cells. We also observed that an ER lacking the LBD (HE15) was a potent constitutive activator of the AP-1 pathway in HeLa, and that this correlated with the ability of these cells to support a large tamoxifen response at the collagenase promoter. To directly test whether transcriptional activation functions were involved in this pathway, we examined the effects of fusing the VP16 activation domain to this receptor (V-ER302C, FIG. 8). FIG. 8B shows that the presence of the VP16 domain greatly potentiated transcription from an ERE, but failed entirely to potentiate transcription activation by ER from the AP-1 site (FIG. 8A). Indeed, the presence of the VP16 domain slightly decreased the activity of HE15 at the AP-1 site. Similar results were also obtained in CHO cells (data not shown). Thus the activation pathway of the LBD deleted receptor at AP-1 sites appears not to respond to exogenous transcriptional activation functions. We argue below that this suggests the existence of an ER pathway that activates transcription from AP-1 sites independent of ER associated transcriptional activation functions.

EXAMPLE 2

Estrogenic Activity Screens

A library of hydroxystilbene derivatives as shown in FIG. 9 was screened for estrogenic activity in cell culture assays using a CAT reporter gene linked to a classical ERE in ERC 1 cells as described in Webb et al., supra. After transient transfection with CAT reporter genes, each hydroxystilbene was added to the cultured ERC1 cells. ER-regulated response was compared either to treatment with 17.beta.-estradiol as a calibration standard or to treatment with an ethanolic vehicle. Hydroxystilbene series 1, 2 and 3 showed no measurable estrogenic activity data (data not shown), whereas series 4 compounds showed weak estrogenic activity (FIG. 10). Of the series 4 compounds 4A, 4E and 4F were found to provide the highest levels of estrogenic activity relative to 17.beta.-estradiol.

To examine whether the series 4 compounds induce estrogenic activity through the ER, we tested the ability of ICI164384 to inhibit the series 4 estrogenic activity. The ICI164384 compound was found to inhibit the estrogenic activity of all the series 4 compounds (FIG. 3). As a negative control, CHO cells, which lack functioning ER, were transfected with the same estrogen-responsive reporter constructs and treated with 17.beta.-estradiol and the series 4 hydroxystilbenes. As expected, no estrogenic activity was seen with these cells.

Dose Response and ER Binding of Series 4

Dose response experiments were performed on the series 4 compounds over a concentration range of 0-100 .mu.M (FIG. 11). For the most active compounds, 4A, 4E, and 4F saturation is observed at 50 .mu.M. The effective concentration that provides 50% maximum activity (EC.sub.50) ranges from -5 .mu.M to .about.15 .mu.M for these three compounds. In vitro ER binding assays were performed on the three most active series 4 compounds to confirm that the estrogenic activity measured in the bioassay correlated with binding affinity for the ER. The inactive hydroxystilbene analog 3D was included in the binding assay as a negative

control. The ER-binding results for compounds 4A, 4E and 4F are consistent with the estrogenic bioassay, as each of the compounds show IC.sub.50 values of 1-10 .mu.M for ER binding (FIG. 12). The two most active compounds in the bioassay, 4A and 4F, also show the highest affinity (1 .mu.M) for the ER, although this affinity is approximately four orders of magnitude lower than that of 17.beta.-estradiol. The analog 3D which showed no activity in the bioassay also shows no binding affinity for the ER.

Additional experiments were performed to characterize further the estrogenic activity of the series 4 compounds identified in the initial screen. Four of the compounds (4A, 4B, 4E and 4F) showed clear dose response profiles over a concentration range of 0-100 .mu.M (FIG. 10). The three most active compounds, 4A, 4E and 4F, show maximum activity at 50 .mu.M and have EC.sub.50 values for estrogen response in the range of 5-15 .mu.M. Three lines of evidence suggest that the series 4 estrogenic response is mediated by direct binding of the hydroxystilbene to the estrogen binding site of the ER. First, the fact that no estrogenic activity was observed in reporter gene-transfected CHO cells which lack a functional ER provides evidence that the response to the series 4 compounds was ER-mediated. Second, the observation that the response initiated by all the series 4 compounds could be inhibited by ICI 164384, a potent steroidal antiestrogen that competes with 17 .beta.-estradiol for binding to the ER, provides evidence that the hydroxystilbenes act through direct binding to the steroid binding site on the ER. Third, the binding affinity of the series 4 compounds to the ER was directly measured in a competition binding assay with 17 .beta.-estradiol and the measured IC.sub.50 values for the series 4 hydroxystilbenes correlate approximately with the EC.sub.50 values measured from the dose response bioassay.

Structure-activity relationships (SAR) in both the hydroxy-substituted and distal aromatic rings of the hydroxystilbenes are evident from the varying estrogenic activities of the library. Based on the observation that activity was only seen in the series 4 compounds, it appears that a para orientation between the hydroxyl substituent and the stilbene olefin is a requirement for ER-binding and activation. The para orientation is sensitive to additional substitution as evidenced by the fact that no estrogenic activity was observed for the series 3 (4-hydroxy-3-nitro) compounds; it is unclear whether steric or electronic factors are responsible for the lack of activity in series 3. For the series 4 compounds, the three most active hydroxystilbenes bear either small fluorine substituents (4E, 4F) or no substitution (4A) in the distal aromatic ring. This distal ring SAR is somewhat subtle: based on the dose-response data, the 4'-Br substituted compound (4C) shows almost no activity, whereas the 4'-F substituted compound (4F) is the second most active member of the series. Here again, it is unclear whether steric effects, electronic effects, or a combination of both are responsible for the variations in estrogenic activity.

Conclusion

The above results show that three of the hydroxystilbene analogs permeate cell membranes and trigger a dose-dependent estrogenic response with EC.sub.50 values in the range of 5-15 .mu.M. Results from competition-response and ER-binding experiments with the antiestrogen IC1164384 and 17 .beta.-estradiol provide evidence that the non-steroidal hydroxystilbene analogs elicit the estrogenic response through direct interaction with the steroid binding site of the estrogen receptor. In addition, structure-activity relationships for the hydroxystilbene pharmacophore are evident from the activity profile of the library. Such information could prove useful for predicting potential estrogenic activity of environmental pollutants and pharmaceuticals.

The above examples are provided to illustrate the invention but not to limit its scope. Other variants of the invention will be readily apparent to one of ordinary skill in the art and are encompassed by the appended claims. All publications, patents, and patent applications cited herein are hereby incorporated by reference.

SEQUENCE LISTING (1) GENERAL INFORMATION: (iii) NUMBER OF SEQUENCES: 1 (2) INFORMATION FOR SEQ ID NO:1: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 15 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear (ii) MOLECULE TYPE: DNA (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1: AGGTCACAGTGACCT15

CLAIMS:

What is claimed is:

1. A method for screening a test compound known to have antiestrogenic activity for agonistic estrogenic activity mediated through an indirect estrogen response, the method comprising:

a) providing a cell comprising AP1 proteins, an estrogen receptor, and a construct comprising a promoter comprising an AP1 site which regulates expression of a reporter gene;

b) contacting said cell with said test compound known to have antiestrogenic activity; and

c) detecting the expression of said reporter gene wherein enhanced expression of said reporter gene indicates that said test compound has agonistic estrogenic activity mediated through an indirect estrogen response.

2. The method of claim 1, wherein the cell is an Ishikawa cell.

3. The method of claim 1, wherein the cell is genetically engineered to express the estrogen receptor at higher levels than said cell without the genetic engineering.

4. The method of claim 3, wherein said cell is an ERC1 cell.

5. The method of claim 1, wherein the promoter is genetically engineered to comprise an AP1 site.

6. The method of claim 1, wherein the cell is derived from uterine tissue.

7. The method of claim 6, wherein the cell is a HeLa cell or an Ishikawa cell.

8. The method of claim 1, wherein said antiestrogenic activity is determined by a method comprising the steps of:

a) providing a second cell comprising an estrogen receptor and a construct comprising a promoter comprising a standard estrogen response element which regulates the expression of a second reporter gene;

b) contacting said second cell with said test compound and a second compound known to have agonistic estrogenic activity mediated through a direct estrogenic response; and

c) detecting the expression of said second reporter gene, wherein inhibition of expression of said second reporter gene produced by said compound known to have agonistic estrogenic activity mediated through a direct estrogenic response indicates that said test compound has antiestrogenic activity.

9. The method of claim 8, wherein the response element is from the Xenopus vitellogenin A2 gene.

10. The method of claim 1, wherein said cell further comprises a construct comprising a promoter comprising a standard estrogen response element which regulates expression of said second reporter gene.

11. The method of claim 10 wherein said standard estrogen response element Is from the Xenopus vitellogenin A2 gene.

12. A method for screening a test compound for the ability to inhibit agonistic estrogenic activity mediated through an indirect estrogen response, the method comprising:

a) providing a cell comprising AP1 proteins, an estrogen receptor, and a construct comprising a promoter comprising an AP1 site which regulates expression of a reporter gene;

b) contacting said cell with said test compound and a compound known to have agonistic estrogenic activity mediated through an indirect estrogen response; and

c) detecting the expression of said reporter gene wherein inhibition of enhanced expression of said reporter gene produced by said compound known to have agonistic estrogenic activity mediated through an indirect estrogen response indicates that said test compound inhibits agonistic estrogenic activity mediated

through an indirect estrogen response.

13. The method of claim 12, wherein the compound known to have agonistic estrogenic activity mediated through an indirect estrogen response is tamoxifen.

14. The method of claim 12, wherein the cell is genetically engineered to express the estrogen receptor at higher levels than said cell without the genetic engineering.

15. The method of claim 14, wherein said cell is an ERC1 cell.

16. The method of claim 12, wherein the promoter is genetically engineered to comprise an AP1 site.

17. A method for screening a test environmental compound for agonistic estrogenic activity mediated through an indirect estrogen response, the method comprising:

a) providing a cell comprising AP1 proteins, an estrogen receptor, and a construct comprising a promoter comprising an AP1 site which regulates expression of a reporter gene;

b) contacting said cell with said test environmental compound, and

c) detecting the expression of said reporter gene wherein enhanced expression of said reporter gene indicates that said test environmental compound has agonistic estrogenic activity mediated through an indirect estrogen response.

18. The method of claim 17, wherein said cell further comprises a construct comprising a promoter comprising a standard estrogen response element which regulates expression of a second reporter gene.

19. The method of claim 17, wherein the reporter gene is CAT.

20. The method of claim 17, wherein the cell is genetically engineered to express the estrogen receptor at higher levels than said cell without the genetic engineering.

21. The method of claim 17, wherein the cell is an ERC1 cell.

22. A method for screening a test compound for the ability to activate transcription mediated through an indirect or direct estrogen response, the method comprising:

a) providing a cell comprising AP1 proteins, an estrogen receptor, and a construct comprising a promoter comprising an AP1 site which regulates expression of a first reporter gene;

b) contacting said cell with said test compound;

c) detecting the expression of said first reporter gene wherein enhanced expression of said first reporter gene indicates that said test compound has agonistic estrogenic activity mediated through an indirect estrogen response;

d) providing a second cell comprising an estrogen receptor and a construct comprising a promoter comprising a standard estrogen response element which regulates expression of a second reporter gene;

e) contacting said second cell with said test compound; and

f) detecting the expression of said second reporter gene wherein enhanced expression of said second reporter gene indicates that said test compound has agonistic estrogen activity mediated through a direct estrogen response.

23. The method of claim 22, wherein said first cell is an Ishikawa cell.

24. The method of claim 22, wherein said first cell is genetically engineered to express estrogen receptors at a higher level than the same cell prior to said genetic engineering.

25. The method of claim 22, wherein said first cell is derived from uterine tissue.

26. The method of claim 25, wherein the cell is a HeLa cell or an Ishikawa cell.

27. The method of claim 22, wherein said standard estrogen response element is from the Xenopus vitellogenin A2 gene.